

Advanced glycation end-products as mediators of the aberrant crosslinking of extracellular matrix in scarred liver tissue

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The extracellular matrix of cirrhotic liver tissue is highly crosslinked. Here we show that advanced glycation end-products (AGEs) mediate crosslinking in liver extracellular matrix and that high levels of crosslinking are a hallmark of cirrhosis. We used liquid chromatography–tandem mass spectrometry to quantify the degree of crosslinking of the matrix of decellularized cirrhotic liver samples from patients and from two mouse models of liver fibrosis and show that the structure, biomechanics and degree of AGE-mediated crosslinking of the matrices can be recapitulated in collagen matrix crosslinked by AGEs *in vitro*. Analyses via cryo-electron microscopy and optical tweezers revealed that crosslinked collagen fibrils form thick bundles with reduced stress relaxation rates; moreover, they resist remodelling by macrophages, leading to reductions in their levels of adhesion-associated proteins, altering HDAC3 expression and the organization of their cytoskeleton, and promoting a type II immune response of macrophages. We also show that rosmarinic acid inhibited AGE-mediated crosslinking and alleviated the progression of fibrosis in mice. Our findings support the development of therapeutics targeting crosslinked extracellular matrix in scarred liver tissue.

Tissue injury can result in fibrotic disease, eventually leading to lethal cirrhosis in liver^{1,2}. The crosslinking of the extracellular matrix (ECM) is one of the critical characteristics of liver cirrhosis, which contributes to the formation of scar tissue. Recovery from fibrosis becomes very difficult in the cirrhotic stage, largely owing to the degree of ECM crosslinking^{3,4}. The most widely reported mechanism for ECM crosslinking in liver fibrosis is mediated by lysyl oxidase (LOX), preliminarily via the formation of pyridinoline (PYD)-based crosslinks between collagen molecules⁵ (Fig. 1a). Unfortunately, clinical trials of the foremost

LOX-targeting molecule, simtuzumab, showed no efficacy in the treatment of liver cirrhosis^{6,7}. This result suggested that other crosslinking mechanisms, in addition to LOX, may also contribute to the formation of cirrhotic ECM, in particular mechanisms mediated by transglutaminase (TGM) and advanced glycation end-products (AGE) (Fig. 1a).

So far, no evidence has revealed the role of ECM crosslinking mediated by AGE in liver cirrhosis. The major factors that drive AGE crosslinking are reducing sugars^{8,9}, such as glucose, which are reportedly dysregulated in cirrhotic liver due to impaired liver function^{10,11}.

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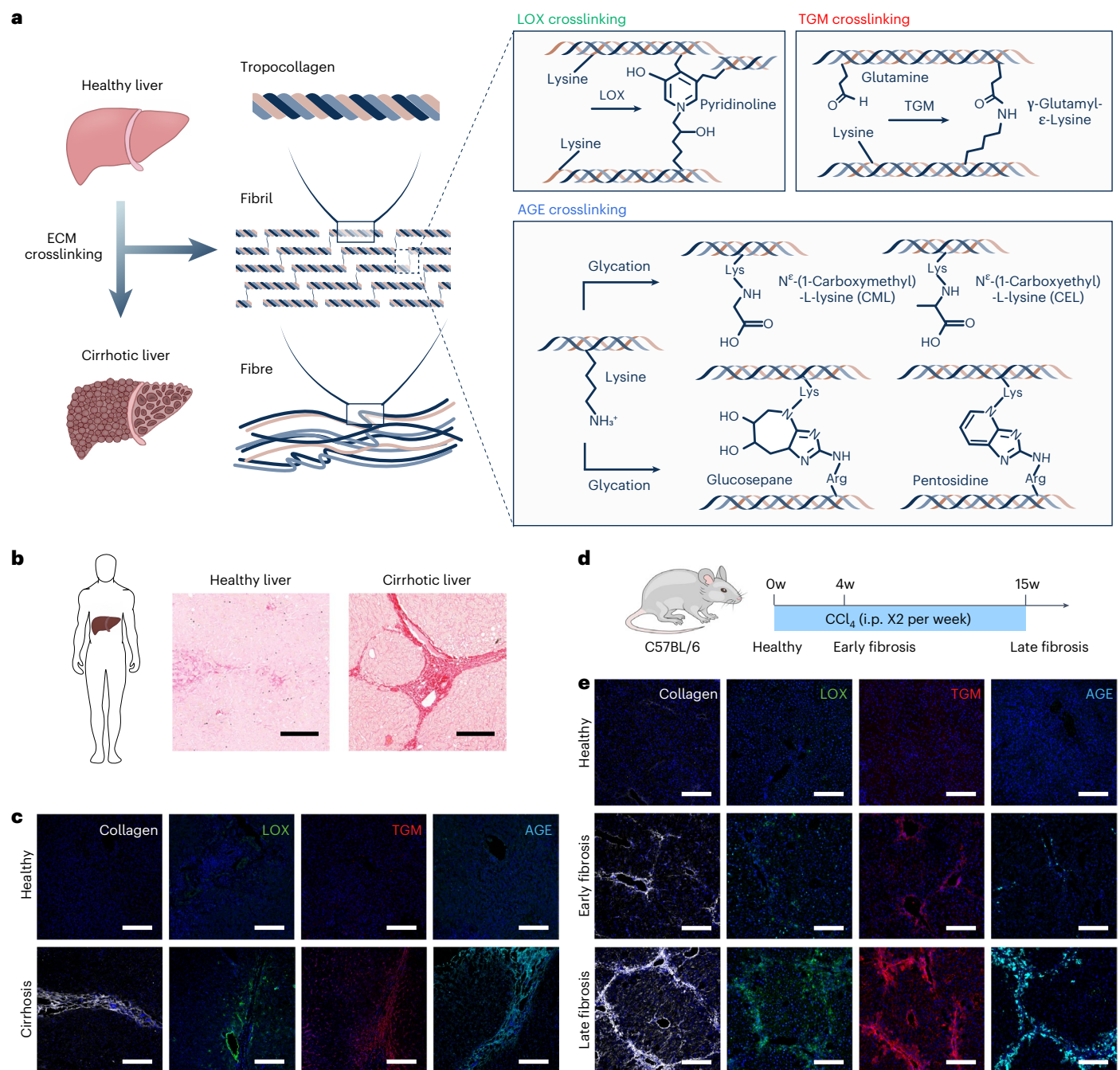


Fig. 1 | Liver cirrhosis is accompanied by ECM crosslinking. **a**, Schematic of critical crosslinking mechanisms, namely LOX, TGM and AGE crosslinking, in ECM during liver fibrosis progression. **b**, Scar tissue stained by Sirius red assay in liver samples of patients with established cirrhosis. The healthy and cirrhotic liver samples used here were derived from human #10 and patient #9, respectively, as indicated in Supplementary Table 1. Scale bars, 200 μm. **c**, Representative images of collagen and the mediators of ECM crosslinking in clinical liver samples

from healthy human and cirrhotic patients. Scale bars, 200 μm. **d**, Schematic of establishment of CCl₄-induced mouse liver fibrosis model, including healthy, early fibrosis and late fibrosis stages. **e**, Representative images of collagen and the mediators of ECM crosslinking in liver tissue samples obtained from mice with different liver fibrosis stages. Co-staining of AGE and collagen in the late-fibrotic tissue is shown in Supplementary Fig. 3c. Scale bars, 200 μm.

Up to 30% of cirrhotic patients suffer from diabetes, and severe cirrhosis is usually accompanied by glucose metabolism disorders or diabetes complications^{1,2,12,13}, which suggests the increased accessibility of liver ECM to glucose, thus resulting in highly AGE-crosslinked ECM. The formation of AGE-crosslinked ECM usually requires a highly complex chemical reaction process in which a variety of crosslinking structures are formed¹⁴. The essential ones include monovalent AGE, namely N^ε-(1-carboxymethyl)-L-lysine (CML) and N^ε-(1-carboxyethyl)-L-lysine

(CEL), and bivalent AGE, namely glucosepane and pentosidine^{15,16} (Supplementary Fig. 1). This diversity of crosslinking structures also poses a challenge for the quantitative study of AGE-crosslinked ECM. Moreover, some in vitro findings of AGE-crosslinked ECM are of limited relevance for guiding clinical studies due to insufficient connections with in vivo conditions^{17,18}.

ECM crosslinking has been reported to produce pathological mechanical cues, such as increased ECM stiffness and altered

viscoelasticity, which aberrantly regulates cellular behaviours and consequently results in fibrosis exacerbation^{8,19}. Notably, since the single fibril is the basic unit of natural ECM with fibrous structure, the properties of fibrils within the bulk ECM are vital to mediating the cell–matrix interaction^{20,21}. Detailed studies focusing on how crosslinking affects the structure and viscoelasticity of ECM at the single-fibril level could thus reveal the underlying pathogenic roles of these aberrant biomechanical cues.

Macrophages act as central regulators of liver fibrosis, displaying high phenotypic diversity during fibrosis progression²², which could be affected by biomechanical cues of the ECM^{23,24}. Elevated response of macrophages to T helper 2 cell-associated cytokines (that is, type II immune response of macrophages) substantially promotes the progression of liver fibrosis^{25–27}. Definitive evidence is needed to clarify the mechanisms by which ECM crosslinking could regulate macrophage response.

In this work, we developed a method, which we named ‘absolute quantification of matrix-specific crosslinking’ (AQMC), that uses liver tissue decellularization in conjunction with liquid chromatography–tandem mass spectrometry (LC–MS/MS) to absolutely quantify the degree of crosslinking in the ECM without being disturbed by non-ECM components. We found that highly AGE-crosslinked ECM was a hallmark of liver cirrhosis by quantifying crosslinking degree in clinical samples and two animal models. To explore the characteristics of AGE-crosslinked ECM, we reconstructed AGE-crosslinked collagen matrices *in vitro*, which recapitulated the characteristics of *in vivo* fibrotic ECM caused by AGE crosslinking, as reflected in the comparable crosslinking degree and biomechanics. Cryogenic transmission electron microscopy (cryo-EM) and optical tweezer techniques were used to investigate the structure and viscoelasticity of AGE-crosslinked collagen matrix at the single-fibril level, and revealed a decrease in the stress relaxation rate of AGE-crosslinked fibrils. Furthermore, AGE-crosslinked fibrils showed resistance to macrophage-mediated remodelling and contributed to aberrant mechano-responsiveness of macrophages, resulting in increased type II immune response. Targeting AGE-crosslinked ECM with rosmarinic acid was effective both *in vitro* and *in vivo*, and was accompanied by alleviation of late liver fibrosis *in vivo*, suggesting that AGE crosslinking could serve as a potential intervention target in cirrhosis treatment.

Results

Liver cirrhosis is accompanied by increased ECM crosslinking

To verify whether ECM crosslinking occurs in liver cirrhosis, we examined the expression of essential mediators of crosslinking, including LOX, TGM and AGE, in liver tissue from cirrhosis patients (Fig. 1a–c). The healthy liver tissue showed lobular structure with hardly any collagen tissue stained by Sirius red. In contrast, the formation of collagen scar could be observed in human cirrhotic liver samples, destroying the lobular structure (Fig. 1b and Supplementary Fig. 2). Meanwhile, all the crosslinking mediators were highly expressed in the cirrhotic tissue samples, mainly in the inter-lobular regions (Fig. 1c). Similar results were observed in samples from different stages of liver fibrosis from a CCl₄-induced liver fibrosis mouse model, including the

healthy stage, the early stage of liver fibrosis (F₁, estimated by METAVIR system²⁸) and the late stage of liver fibrosis (F₃–F₄) (Fig. 1d and Supplementary Fig. 3). The expression of collagen and all these mediators of crosslinking was gradually upregulated during fibrosis progression, with these mediators mainly localized in the peri-lobular regions (Fig. 1e). The above results suggest that liver cirrhosis is accompanied by ECM crosslinking; moreover, crosslinking mediated by AGE and TGM, in addition to LOX, could all potentially contribute to modulating the properties of liver ECM.

AQMC identifies AGE-crosslinked ECM in liver cirrhosis

To determine the extent to which these three crosslinking mechanisms contribute to ECM crosslinking in liver cirrhosis, we developed the AQMC method by taking advantage of liver decellularization and LC–MS/MS (Fig. 2a and Extended Data Fig. 1). We used a whole-liver-perfusion decellularization method that effectively separated the ECM from cells in mouse liver and also non-significantly affected the collagen composition and structural properties of the liver ECM (Extended Data Fig. 1b,c and Supplementary Fig. 4). After serial digestion, the crosslinks could be completely isolated from the liver ECM, followed by detection using LC–MS/MS. The crosslinking degree was defined as the molar ratio of crosslinks to tropocollagen molecules. Using our AQMC method, representative crosslinks generated by LOX, TGM and AGE crosslinking were all detectable in the decellularized ECM (that is, pyridinoline for LOX crosslinking; γ -Glutamyl- ϵ -Lysine for TGM crosslinking; CML, CEL, glucosepane and pentosidine for AGE crosslinking) (Extended Data Fig. 2).

By using the AQMC method, we were able to characterize the ECM crosslinking degree in liver samples from clinical patients with established cirrhosis. Surprisingly, AGE crosslinking showed the highest crosslinking degree in liver ECM of cirrhosis patients, followed by TGM crosslinking and LOX crosslinking. CML contributed the most to AGE crosslinking, followed by CEL, glucosepane and pentosidine (Fig. 2b).

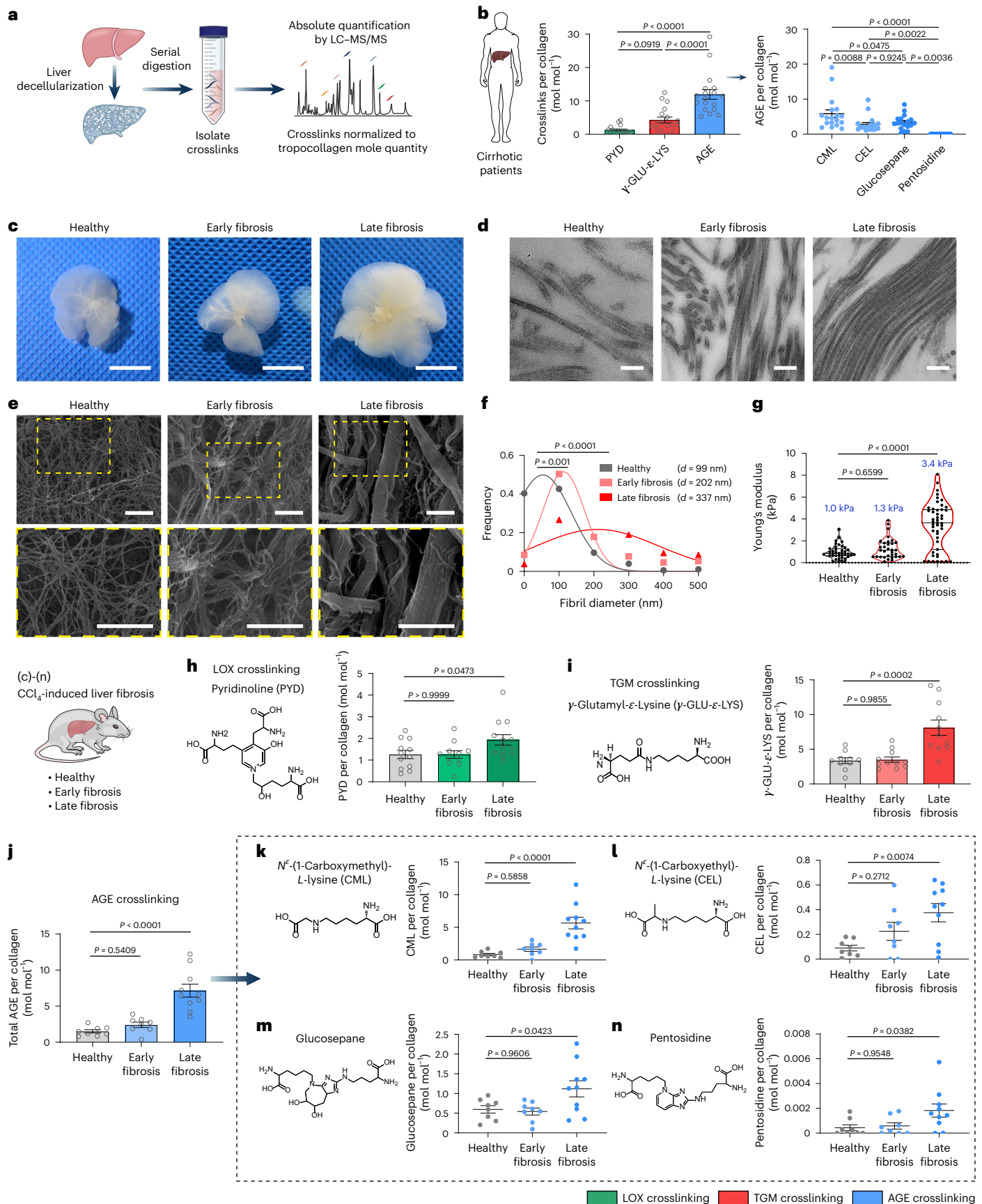
We then sought to determine ECM crosslinking, as well as its potential effects on characteristics of liver ECM in fibrotic animal models. In the CCl₄-induced mouse liver fibrosis model, the ECM obtained by decellularization retained its original liver tissue morphology. The healthy ECM was white and translucent, whereas the late-fibrotic ECM was non-transparent, indicating a dense internal architecture (Fig. 2c). The decellularized liver ECM obtained from whole liver in late-fibrotic mice was substantially heavier than that obtained from healthy mice, indicating increased ECM deposition along with fibrosis progression (Supplementary Fig. 3h). Both transmission electron microscopy (TEM) and scanning electron microscopy (SEM) images showed that collagen fibrils in healthy ECM were fine and uniform (Fig. 2d,e). With increasing progression of liver fibrosis, a greater number of the fine fibrils were tightly attached, intertwined and eventually formed thick fibril bundles in late-fibrotic ECM (Fig. 2e,f). The length of D-period stripes decreased with longer duration of fibrosis progression (Supplementary Fig. 3i). Moreover, the elastic modulus of decellularized liver ECM increased with fibrosis progression (Fig. 2g).

Fig. 2 | AQMC identifies AGE crosslinking of ECM as a critical feature of liver cirrhosis. **a**, Schematic of the AQMC method. Details are shown in Methods and illustrated in Extended Data Fig. 1. **b**, Quantification of crosslinking degree of liver ECM from patients with established liver cirrhosis ($n = 18$ pieces of liver decellularized ECM obtained from 9 patients). Left: the degree of crosslinking mediated by LOX, TGM and AGE. Right: the degree of crosslinking mediated by different types of AGE. **c**, Representative bright-field images of liver decellularized ECM obtained from mice with different extents of CCl₄-induced liver fibrosis. Scale bars, 1 cm. **d**, Representative TEM images of mouse liver decellularized ECM. Scale bars, 200 nm. **e**, Representative SEM images of mouse liver decellularized ECM with fibrous structure. Scale bars, 2 μ m. **f**, Statistical analysis of fibril diameter in mouse liver decellularized ECM ($n \geq 105$ fibrils

randomly selected from at least 5 fields of SEM images). **g**, Young's modulus of mouse liver decellularized ECM measured by atomic force microscopy (AFM) ($n \geq 33$ randomly selected points of measurement per group). **h–n**, Quantification of crosslinking degree of liver ECM from mice with different extents of CCl₄-induced liver fibrosis. Sample sizes indicate the number of pieces of liver decellularized ECM obtained from at least 4 mice for each condition. **h**, LOX crosslinking degree quantified by pyridinoline ($n \geq 11$); **i**, TGM crosslinking degree quantified by γ -Glutamyl- ϵ -Lysine ($n \geq 10$); **j**, AGE crosslinking degree quantified by CML (**k**), CEL (**l**), glucosepane (**m**) and pentosidine (**n**) ($n \geq 8$). Statistical analysis was performed using one-way ANOVA with Turkey's test. Results are presented as mean $\pm$ s.e.m.

Comparative analysis of the crosslinking degree of liver ECM in CCl₄-induced liver fibrosis model revealed that the extent of LOX crosslinking did not increase in the early stage of fibrosis but only

slightly increased by ~1.5-fold in the late fibrosis stage (Fig. 2h). The degree of TGM crosslinking was non-significantly changed in early fibrosis relative to the healthy stage, but was considerably elevated



by 2.4-fold in late fibrosis (Fig. 2i). Similarly, AGE crosslinking was non-significantly changed in early fibrosis, but was dramatically increased by 4.6-fold (relative to the healthy stage) in the late-fibrotic ECM (Fig. 2j). Among all types of AGE crosslinks, CML showed the highest expression level in late-fibrotic ECM, followed by CEL, glucosepane and pentosidine (Fig. 2k–n).

The essential role of AGE crosslinking in liver cirrhosis was also proven in another late-fibrosis model (HF fibrosis) induced by a high-fat, choline-deficient, amino acid-defined diet (HFDCAA diet) that mimicked the pathogenesis of human non-alcoholic liver fibrosis^{29,30} (Extended Data Fig. 3). Similar to the CCl₄-model, the ECM of livers from the HF-fibrotic mice obtained by decellularization showed dense fibril structure (Extended Data Fig. 3c,d). Quantification of ECM crosslinking by AQMC also showed a high degree of both AGE crosslinking and LOX crosslinking in HF-fibrotic liver ECM. In contrast, TGM crosslinking was substantially decreased (Extended Data Fig. 3e–j).

These results provide strong evidence that a high degree of AGE crosslinking is a hallmark of the ECM in liver cirrhosis, which was verified in clinical samples and two mouse liver fibrosis models. Compared with LOX and TGM crosslinking, AGE crosslinking showed the highest increase over the course of fibrosis progression in the CCl₄-induced fibrosis model.

In vitro reconstruction of AGE-crosslinked collagen matrix

To investigate how AGE crosslinking affects the biomechanical properties of the ECM in vitro, we reconstructed biomimetic collagen matrix models with a tuneable degree of AGE crosslinking by using glucose, a primary mediator of AGE crosslinking in vivo, and quantified AGE crosslinking in the collagen matrix by the AQMC method (Fig. 3a). By controlling the reaction condition for crosslinking, we prepared AGE-crosslinked collagen matrix with relatively low and high crosslinking degree (that is, AGE^{lo/hi}-crosslinked matrix) evident in the increased level of all the typical AGE crosslinks (Fig. 3b–f). Rosmarinic acid (RA) is a natural phenolic acid that can reportedly inhibit the chemical reactions required for AGE crosslinking^{31,32} (Supplementary Fig. 1). We found that the addition of RA to the AGE^{hi} crosslinking treatment resulted in a decrease in the crosslinking degree of collagen matrix, similar to that of non-crosslinked matrix. RA treatment exhibited non-significant effects on non-crosslinked matrix, and RA did not directly react with the glucose, indicating the specificity of its inhibitory effect on the AGE crosslinking reaction (Supplementary Figs. 5 and 6). We therefore selected the AGE^{hi}-crosslinked matrix for subsequent in vitro assays due to the comparable degree of AGE crosslinking with in vivo late-fibrotic liver ECM in the CCl₄-induced model (Fig. 3b).

SEM images showed that collagen fibrils in non-crosslinked collagen matrix displayed a relatively fine and uniform structure, which was similar to fibrils in healthy liver ECM (Fig. 3g,h). AGE crosslinking triggered close alignment and intertwining of the fine fibrils, resulting in formation of thick fibril bundles—a characteristic of ECM in late-fibrosis liver tissue. This effect was inhibited by RA treatment (Fig. 3g,h). AGE-crosslinked matrix also exhibited a higher elastic modulus than non-crosslinked matrix, which was consistent with in vivo changes in the ECM during fibrosis progression (Fig. 3i).

Cryo-EM was used for detailed examination of alterations in the collagen fibril structure. A tilt series of fibrils acquired by cryo-EM tomography was used for three-dimensional (3D) reconstruction (Fig. 3j,k, and Supplementary Fig. 7 and Videos 1–3). Analyses of collagen fibril models confirmed that AGE crosslinking resulted in intertwining of the collagen fibrils to form tangled fibril bundles, indicating that inter-fibril interactions were strengthened after formation of AGE crosslinks between adjacent fibrils (Supplementary Video 2). However, these strengthened interactions were inhibited by exposure to RA as indicated by uniform and untwisted fibrils, similar to those of non-crosslinked collagen fibrils (Supplementary Video 3).

By analysing electron density profiles of collagen fibrils, we found a decreased length of the D-period in AGE-crosslinked fibrils compared with that in non-crosslinked fibrils, including an increased length of the overlap region and an increased ratio of the overlap length to D-period length (O/D ratio) (Fig. 3l–p). These changes in D-period morphology suggested increased intra-fibril interactions caused by AGE crosslinking, indicating a more close-packed arrangement of tropocollagen molecules within a fibril, which is consistent with the feature of fibrils in late-fibrotic ECM in vivo (Supplementary Fig. 3i). These alterations in the D-period pattern were diminished by exposure to RA as well. These changes in intra-fibril interactions could be explained by a theoretical model based on analysis of previously reported AGE crosslinking sites on tropocollagen molecules³³ (Supplementary Fig. 8).

Taken together, the reconstructed AGE-crosslinked collagen matrix recapitulates the essential characteristics of late-fibrotic liver ECM in vivo as evidenced by the similarity in AGE crosslinking degree, the increased elastic modulus of the matrix and the formation of thick fibril bundles.

Crosslinking inhibits stress relaxation of collagen fibrils

Since a single fibril is the basic unit of fibrous ECM, the properties of fibrils within the bulk ECM are vital to mediating the cell–matrix interaction²⁰. Therefore, we investigated changes in collagen viscoelasticity related to AGE crosslinking from the bulk collagen matrix to the single-fibril levels to characterize the role of crosslinking in pro-disease mechanotransduction.

We probed the matrix stiffness and the stress relaxation rate of the bulk collagen matrix using the single-trap optical tweezer technique. Bead infused in matrix was manipulated by an optical trap to move along a certain direction (Fig. 4a). The matrix stiffness was calculated according to the slope of the force-displacement curve in linear extent. The bead was then kept static for a period of time to record force decay. Stress relaxation for the collagen matrix was quantified by the time it took for the normalized force to reduce to one-half its peak value ($\tau^{1/2}$, half relaxation time). The AGE-crosslinked collagen matrix showed substantial increases in stiffness and stress relaxation time compared with the non-crosslinked matrix, indicating that AGE-crosslinked matrix was more difficult to remodel (Fig. 4b–d).

The challenges in probing the viscoelasticity of single collagen fibrils were overcome by an optimized double-trap optical tweezer method. We linked the biotin molecules to triple-helix collagen monomers, followed by preparing a period of collagen fibril bound by two streptavidin-coated beads, which made it possible to stretch a single collagen fibril through manipulation of beads using optical traps (Fig. 4e). Through axial stretching, highly linear force-extension curves for collagen fibrils were obtained that could fit well in a classical worm-like-chain model, indicating the single-fibril characteristics of the samples (Fig. 4f). The accuracy and stability of this system was verified by the stress relaxation test on a pure elastic standard, and results showed no detectable stress relaxation (Fig. 4g). It was found that AGE crosslinking did not change the Young's modulus of collagen fibrils (Fig. 4h). However, the stress relaxation rate was substantially inhibited in AGE-crosslinked collagen fibrils, as seen in a sharply increased $\tau^{1/2}$ value compared with the non-crosslinked group. Moreover, fibrils showed a restored fast stress relaxation rate similar to non-crosslinked fibrils in the presence of RA to inhibit AGE crosslinking (Fig. 4i,j).

These results unveiled the effect of AGE crosslinking on the viscoelasticity of collagen from the bulk-matrix scale to the single-fibril scale, denoting substantially inhibited stress relaxation rates in AGE-crosslinked fibrils.

Crosslinked fibrils resist macrophage-mediated remodelling

Previous work has shown that cells are unable to effectively recruit fibrils with a high elastic modulus, resulting in decreased focal adhesions and inhibited spreading^{20,34}. Considering that

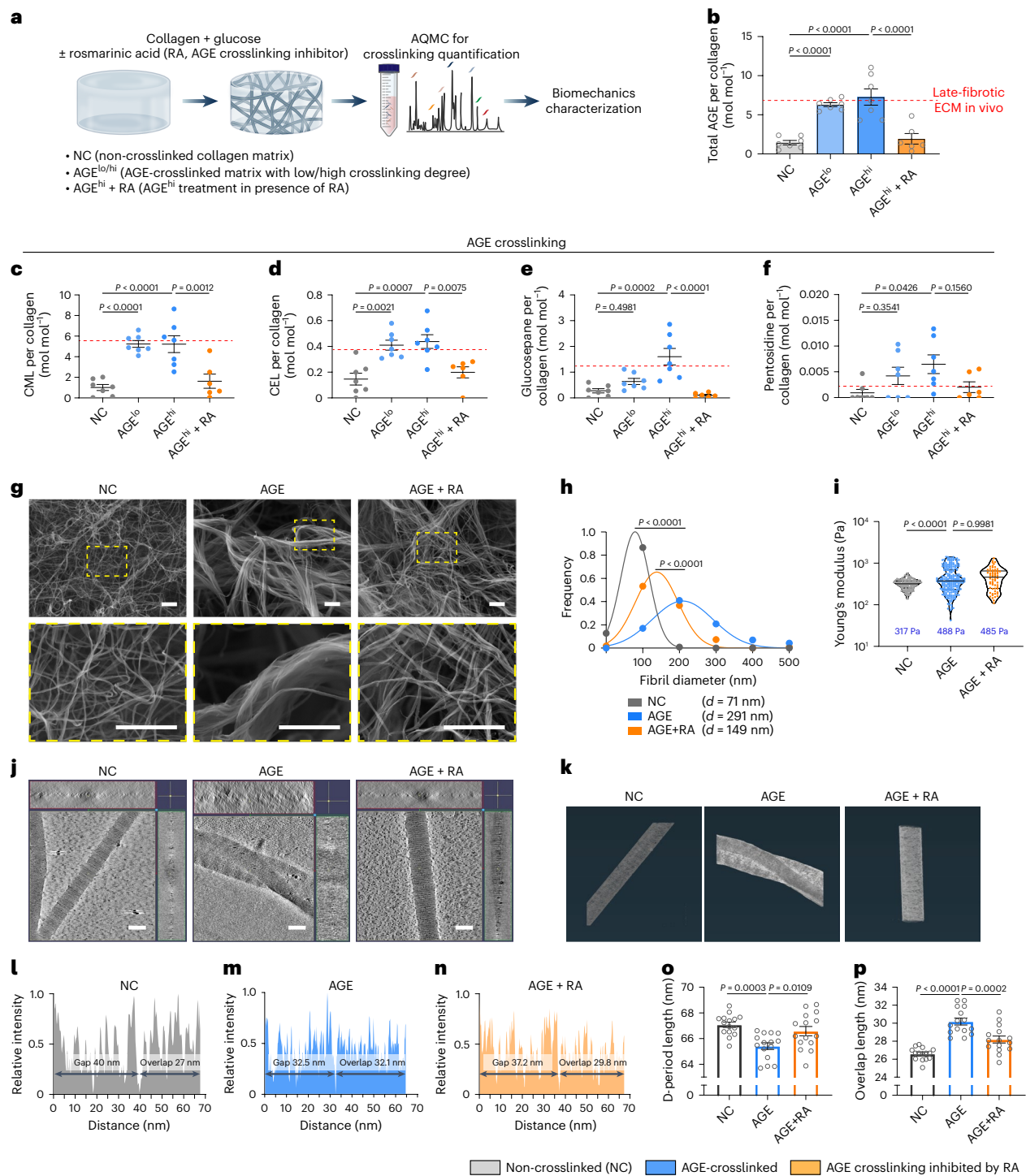


Fig. 3 | In vitro reconstruction of AGE-crosslinked collagen matrix recapitulating in vivo late-fibrotic liver ECM. **a**, Schematic of the reconstruction and characterization of AGE-crosslinked collagen matrix in vitro, utilizing collagen, glucose and RA (a specific inhibitor of AGE crosslinking). RA at a concentration of 100 μ M was used to prepare AGE + RA matrix. **b–f**, Quantification of AGE crosslinking degree in reconstructed collagen matrix. Sample size indicates the independent pieces of collagen matrix. **b**, Total AGE crosslinking degree ($n \geq 6$); **c**, CML ($n \geq 6$); **d**, CEL ($n \geq 6$); **e**, glucosepane ($n \geq 6$); **f**, pentosidine ($n \geq 6$). Red dashed line indicates the AGE crosslinking degree of in vivo liver ECM from mice with late-stage liver fibrosis induced by CCl₄. **g**, Representative SEM images of non-crosslinked matrix (NC), AGE-crosslinked matrix (AGE) and matrix with additional RA to inhibit AGE crosslinking (AGE + RA). Scale bars, 2 μ m. **h**, Statistical analysis of fibril diameter in NC, AGE

and AGE + RA matrix ($n \geq 109$ fibrils randomly selected from at least 5 fields of SEM images). **i**, Young's modulus of NC, AGE and AGE + RA matrix measured by AFM ($n \geq 64$ points of measurement randomly selected from at least 10 fields per sample). **j**, Cryo-electron tomography of collagen fibrils with or without the effect of AGE crosslinking. Scale bars, 100 nm. **k**, Visualization of 3D reconstructed collagen fibrils based on cryo-electron tomography. **l–n**, Intensity profiles of a representative D-period of non-crosslinked collagen fibrils (**l**), AGE-crosslinked collagen fibrils (**m**) and collagen fibrils with additional RA to inhibit AGE crosslinking (**n**). **o**, Statistical analysis of D-period length of collagen fibrils ($n = 15$ randomly selected fibrils). **p**, Statistical analysis of overlap length in D-period stripes of collagen fibrils ($n = 15$ randomly selected fibrils). Statistical analysis was performed using one-way ANOVA with Turkey's test. Results are presented as mean $\pm$ s.e.m.

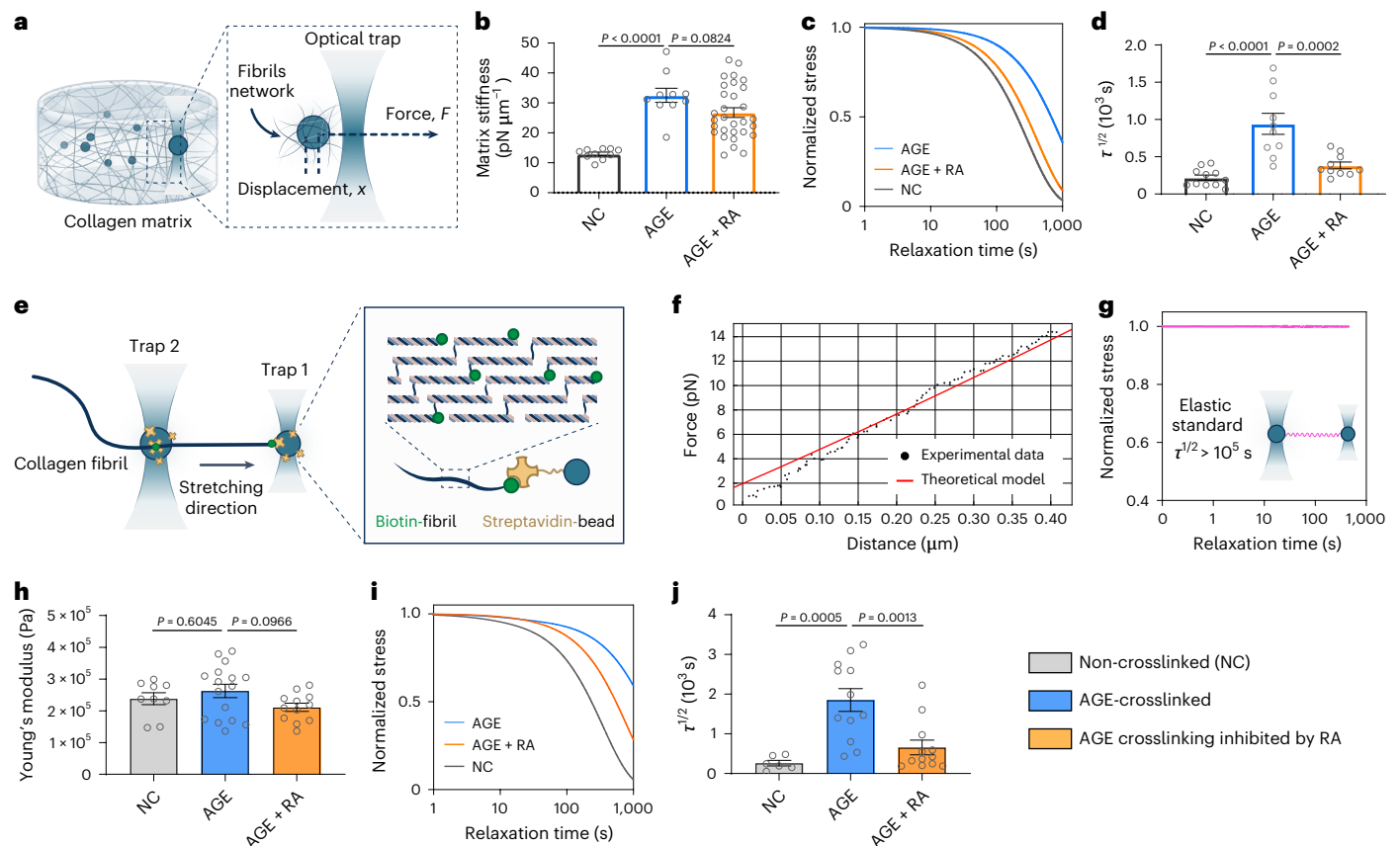


Fig. 4 | AGE crosslinking reduces the stress relaxation rate of collagen matrix at the single-fibril level. **a**, Schematic of viscoelasticity characterization for the collagen matrix using the single-trap optical tweezer technique. **b**, Matrix stiffness of the collagen matrix ($n \geq 10$ points of detection derived from at least 3 independent samples). **c**, Representative stress relaxation curve of the collagen matrix. **d**, Statistical analysis of the timescale at which the stress is relaxed to half its original value, $\tau^{1/2}$, from stress relaxation tests in **c** ($n \geq 10$ points of detection derived from at least 3 independent samples). **e**, Schematic of viscoelasticity characterization for a single collagen fibril using the double-trap optical tweezer

technique. **f**, Representative force-extension curve of stretching a single collagen fibril. The data were fitted to the modified WLC model as detailed in Methods. **g**, The stress relaxation curve of an elastic standard. No significant stress relaxation was detected. **h**, Young's modulus of collagen fibrils ($n \geq 9$ fibrils derived from at least 3 independent samples). **i**, Representative stress relaxation curve of collagen fibrils. **j**, Statistical analysis of $\tau^{1/2}$ from stress relaxation tests of collagen fibrils in **i** ($n \geq 6$ fibrils derived from at least 3 independent samples). Statistical analysis was performed using one-way ANOVA with Turkey's test. Results are presented as mean $\pm$ s.e.m.

AGE-crosslinked fibrils showed decreased stress relaxation rate and could resist remodelling, we next sought to determine how these mechanical cues affected macrophage, an essential regulator for liver fibrosis.

We isolated and seeded bone-marrow-derived macrophages (BMDM) into the non-crosslinked collagen matrix (NC), AGE-crosslinked collagen matrix (AGE) and collagen matrix treated by AGE crosslinking in the presence of RA inhibition (AGE + RA). The reagent residues in collagen matrix, such as endotoxin, were negligible after thorough washing and could not induce unexpected response in the macrophages (Extended Data Fig. 4). The expression of typical genes related to cell-ECM interactions were downregulated in macrophages grown on AGE-crosslinked matrix compared with those grown on non-crosslinked matrix (Supplementary Fig. 9). Notably, macrophages grown on non-crosslinked matrix formed protrusions that were tightly bound to adjacent collagen fibrils, resulting in dense compacted fibril clusters (Fig. 5a). By contrast, macrophages grown on AGE-crosslinked matrix could not form protrusion structures and only adhered to the adjacent fibril bundles, indicating suppressed fibril recruitment and inhibited cell-ECM interactions. In agreement with the established inhibitory effects of RA on fibril viscoelasticity of AGE-crosslinked matrix, we found that the formation of protrusions and fibril recruitment were restored in macrophages grown on AGE + RA matrix (Fig. 5a). To quantify fibril

recruitment by macrophages, we calculated fibril recruitment index scores by normalizing fibril density in the region centred on cells to that of the area without cell remodelling (Fig. 5b,e). We found that a number of collagen fibrils in the non-crosslinked matrix could be recruited to cells and localized to the actin-rich protrusions of macrophages, which was not detectable in AGE-crosslinked matrix. Fibril recruitment was restored in AGE + RA matrix (Fig. 5b,e). Moreover, the decreased fibril recruitment and cell-matrix interactions were indicated by decreased co-localization of cytoskeleton and surrounding collagen fibrils, as observed in macrophages grown on AGE-crosslinked matrix (Supplementary Fig. 10 and Videos 4 and 5).

We then further confirmed the resistance to macrophage-mediated remodelling in AGE-crosslinked collagen fibrils by using optical tweezer assays to simulate the effects of cell-traction forces on recruiting the collagen fibrils (Fig. 5c,d). The applied force was kept constant for a period of time and induced dramatic displacement of non-crosslinked collagen fibrils (Fig. 5d,f and Supplementary Video 6). However, an equivalent force did not move the AGE-crosslinked fibrils to a measurable degree during the stretching period due to the decreased stress relaxation caused by AGE crosslinking. Obvious displacement of fibrils could be observed in the AGE + RA group (Fig. 5d,f, and Supplementary Videos 7 and 8). Taken together, these results demonstrated that AGE-crosslinked collagen fibrils showed

resistance to macrophage-mediated remodelling, which was supported by cell-mediated remodelling assay and cell-traction simulation remodelling assay.

Crosslinked matrix modulates mechanosensing of macrophages

Increased fibril recruitment can promote focal adhesions assembly, which could be characterized by paxillin structures²⁰ and peripheral paxillin localized to actin-rich cell protrusions participating in downstream mechano-regulation³⁵. We observed that macrophages grown on non-crosslinked matrix could form a number of peripheral paxillin adhesions localized in the cell protrusions (Fig. 5g,i). However, fewer peripheral paxillin adhesions were observed in macrophages grown on AGE-crosslinked matrix, and fewer cell protrusions were also observed. Considering that the cytoskeleton is responsible for mechanotransduction of adhesion molecules, we investigated the F-actin organization of macrophages grown on different matrices. Macrophages grown on non-crosslinked matrix showed extending morphology and a high level of F-actin organization compared with those grown on AGE-crosslinked matrix (Fig. 5h and Supplementary Fig. 11). Additionally, the ratio of F-actin to G-actin was lower in macrophages grown on AGE-crosslinked matrix, indicating suppression of actin organization (Supplementary Fig. 11). Consistent with the above results, macrophages grown on AGE + RA matrix exhibited comparable F-actin organization as that of macrophage from non-crosslinked matrix. Macrophages grown on non-crosslinked matrix showed higher cellular stiffness compared with those grown on AGE-crosslinked matrix, which might have resulted from the high-level cytoskeleton organization (Supplementary Fig. 12). To investigate the relationship between the formation of paxillin molecules and cytoskeleton organization in macrophages grown on collagen matrix, we pretreated the macrophages with RGD peptide (a widely reported method to inhibit cell–ECM interactions²¹) before seeding the cells into non-crosslinked collagen matrix. RGD treatment substantially inhibited the paxillin formation in macrophages (Supplementary Fig. 13). Correspondingly, we observed a much lower level of F-/G-actin ratio in RGD-pretreated macrophages grown on collagen matrix compared with the untreated cells.

Actin organization showed a strong association with the epigenetic regulator, HDAC3, the expression of which is responsible for modulating type I and type II immune response of macrophages^{36,37}. By HDAC3 staining, we found that macrophages grown on the non-crosslinked matrix showed a high level of HDAC3 expression and HDAC3 mainly co-localized with the cell nucleus (Fig. 5j–l). In contrast, overall expression and nuclear localization were both decreased in macrophages grown on AGE-crosslinked matrix. Moreover, HDAC3 expression in macrophages grown on AGE + RA matrix was similar to that of the non-crosslinked group. The decreased expression of HDAC3 in macrophages grown on AGE-crosslinked matrix was further validated by reduced protein abundance and inhibited activities compared with those grown on non-crosslinked matrix (Supplementary

Fig. 14). To further investigate whether the decreased HDAC3 was related to the inhibited cytoskeleton organization that was observed in macrophages grown on AGE-crosslinked matrix, we treated the macrophages with increased osmotic pressure, which simulated the physical confinement effect to inhibit cytoskeleton organization^{38,39}. Macrophages grown on 2D glass showed substantially decreased cytoskeleton organization when treated by the increased osmotic pressure. Meanwhile, a much lower level of HDAC3 expression was observed in macrophages along with the decreased cytoskeleton organization (Extended Data Fig. 5a–e). Similarly, the cytoskeleton organization in macrophages grown on non-crosslinked matrix was inhibited by the increased osmotic pressure, which was accompanied by a lower level of HDAC3 expression comparable to that observed in macrophages grown on AGE-crosslinked matrix (Extended Data Fig. 5f–i).

Collectively, these results showed that AGE-crosslinked collagen fibrils exhibited resistance to macrophage-mediated remodelling. The decreased number of recruited fibrils could inhibit the formation of protrusions and paxillin adhesions in macrophages, as well as inhibit cytoskeleton organization and HDAC3 expression.

Crosslinked matrix regulates macrophage immune response

Elevated type II immune response of macrophages are reportedly related to liver fibrosis exacerbation^{25–27}. To verify this effect in our model, we characterized type II immune response in hepatic macrophages isolated from mice with CCL₄-induced liver fibrosis (Supplementary Fig. 15). Flow cytometry analysis showed substantial upregulation of markers for type II immune response in hepatic macrophages isolated from late-fibrotic livers (that is, Arginase1, C-X3-C chemokine motif receptor 1 (CX3CR1), resistin-like molecule alpha (RELMA, encoded by *Retnla*) and transforming growth factor beta (TGFβ)) relative to macrophages isolated from healthy livers (Supplementary Fig. 15c–f). The transcription of these markers was also substantially upregulated (that is, *Arg1*, *Cx3cr1* and *Tgfb1*) (Supplementary Fig. 15g–j), confirming a robust type II immune response of hepatic macrophages in late-fibrotic stage.

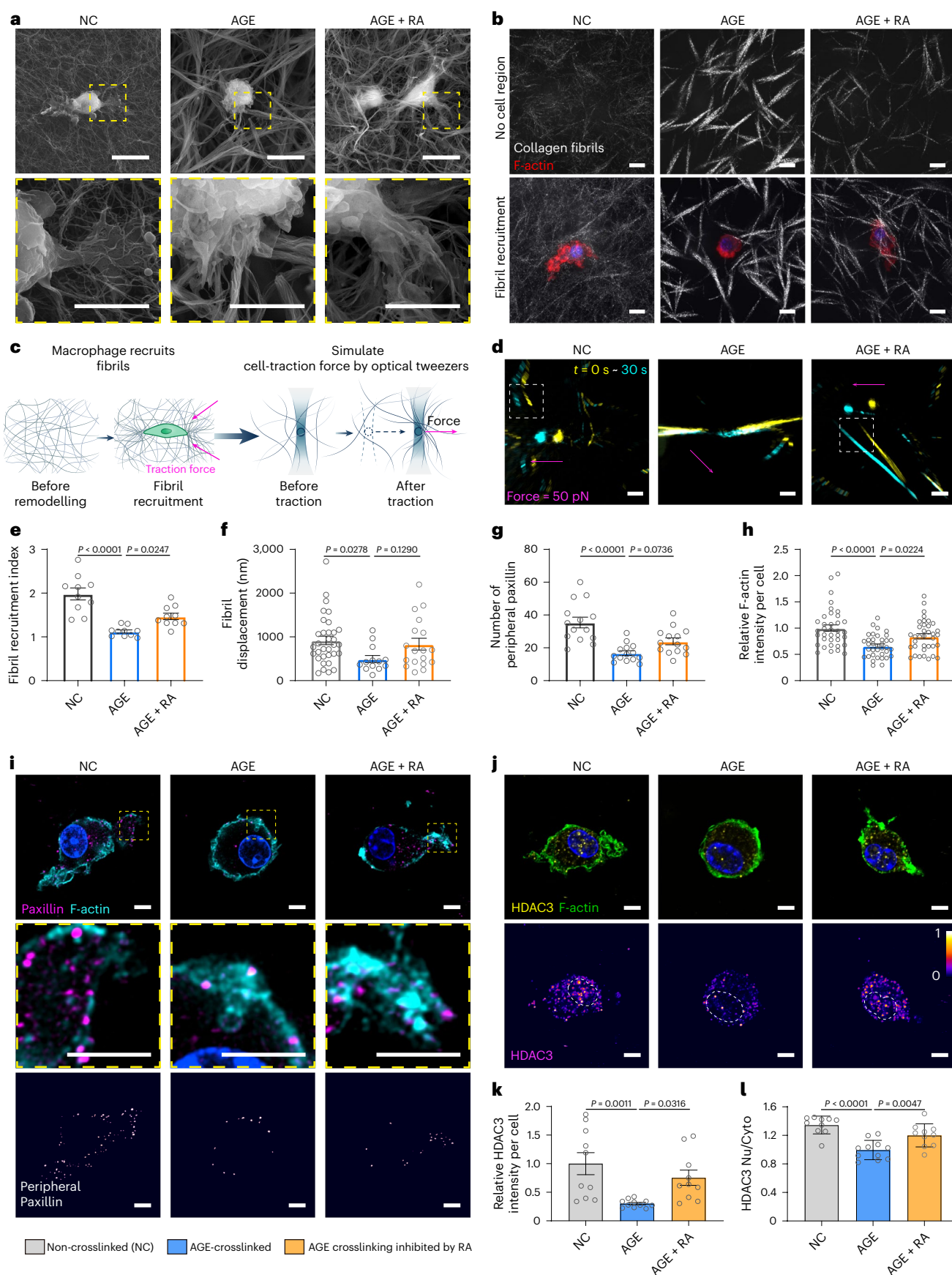
To investigate whether the regulatory effect of AGE-crosslinked matrix on macrophages (that is, decreased adhesion molecules, cytoskeleton organization and HDAC3) were associated with type I and type II immune responses, we examined type I and type II immune responses of macrophages grown on collagen matrix by applying typical lipopolysaccharide (LPS) or interleukin-4 (IL-4) stimulation, respectively (Fig. 6a,c). RNA-seq results showed downregulation of type I immune response-related genes (for example, *Nos2*, *Il6* and *Stat1*) and upregulation of type II immune response-related genes (for example, *Arg1*, *Retnla* and *Cd163*) in macrophages grown on AGE-crosslinked matrix compared with those grown on the non-crosslinked group (Fig. 6b,d). Moreover, matrix metalloproteinase (Mmp) family genes, which show diverse functions in liver fibrosis, maintained low expression in macrophages grown on AGE-crosslinked matrix. The inhibited

Fig. 5 | AGE-crosslinked fibrils show resistance to macrophage-mediated remodelling. **a**, Representative SEM images of macrophages grown on NC, AGE and AGE + RA. Scale bars, 5 μm. **b**, Representative images of collagen fibrils (white) in no-cell region (top) or in cell-centred region being recruited by macrophages (bottom). Macrophages were stained for F-actin (red) and nuclei (blue). Scale bars, 10 μm. **c**, Schematic of collagen fibril stretching using optical tweezer to simulate cell-traction forces that recruit collagen fibrils. The applied force was kept constant for a period of time to record the displacement of fibrils. **d**, Representative images of collagen fibrils before (yellow, $t = 0$ s) and after (cyan, $t = 30$ s) the remodelling effect. The arrows indicate the force directions and the dashed squares indicate the considerable displacement of fibrils. Scale bars, 2 μm. **e**, Statistical analysis of fibril recruitment index in **b** ($n = 10$ cells analysed per condition). **f**, Statistical analysis of fibril displacement after the remodelling as shown in **c** and **d** ($n \geq 13$). **g**, Statistical analysis of the number of peripheral

paxillin per cell as shown in **i** ($n = 14$ cells analysed per condition). **h**, Statistical analysis of the relative intensity of F-actin per cell, as normalized to the non-crosslinked group ($n = 36$ cells analysed per condition). **i**, Representative images of paxillin and F-actin staining. Top: overall view. Middle: detailed view. Bottom: separated channels showing peripheral paxillin that localized with F-actin-rich protrusions. Scale bars, 4 μm. **j**, Representative images of HDAC3 staining in macrophages. Top: HDAC3 (yellow), F-actin (green), cell nuclei (blue). Bottom: colour-coded images of HDAC3. Colour bar indicates pixel intensity values. Cell nuclear outlines are marked by white dashed circles. Scale bars, 4 μm. **k, l**, Statistical analysis of total intensity of HDAC3 per cell (**k**) and nucleus/cytoplasm ratio of HDAC3 (**l**) ($n \geq 10$ cells analysed per condition). Statistical analysis was performed using one-way ANOVA with Turkey's test. Results are presented as mean $\pm$ s.e.m.

type I immune response and promoted type II immune response in macrophages grown on AGE-crosslinked matrix were further confirmed by qPCR assay (Supplementary Fig. 16).

We then isolated cells grown on matrix for flow cytometry analysis. The results showed that a lower proportion of macrophages in the AGE-crosslinked group exhibited high IL-6 and iNOS (inducible nitric



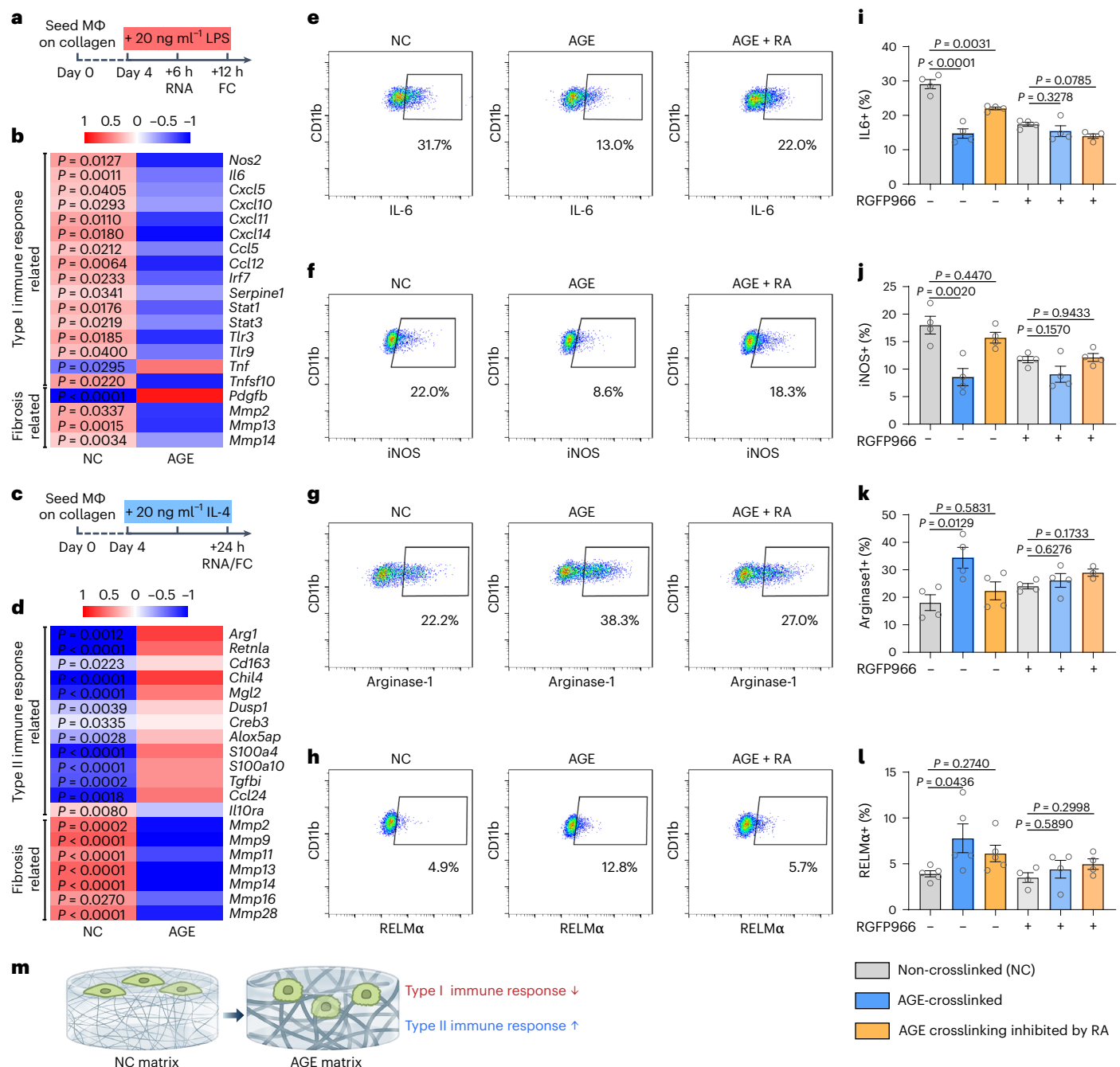


Fig. 6 | Macrophages grown on AGE-crosslinked matrix show downregulated type I response and upregulated type II response. **a**, Schematic of the characterization of the type I immune response of macrophages. **b**, Expression of genes related to the type I immune response of macrophages grown on non- and AGE-crosslinked matrix. Analysed using RNA-seq data ($n \geq 2$ biologically independent samples for sequencing). **c**, Schematic of the characterization of the type II immune response of macrophages. **d**, Expression of genes related to the type II immune response of macrophages grown on non- and AGE-crosslinked matrix. Analysed using RNA-seq data ($n = 3$ biologically independent samples for sequencing). The colour scales indicate the log₂ fold change of genes as detailed in Methods. False discovery rate-adjusted P value of each gene is marked on the plots. $P < 0.05$ is considered statistically significant. **e, f**, Representative flow cytometry plot of IL-6 (e) and iNOS (f) expression in macrophages, assessed

according to the strategy shown in **a**. Corresponding statistical analyses are shown in **i** and **j**. **g, h**, Representative flow cytometry plot of Arginase-1 (g) and RELMα (h) expression in macrophages, assessed according to the strategy shown in **c**. Corresponding statistical analyses are shown in **k** and **l**. **i–l**, Flow cytometry analysis of macrophages' type I and type II immune responses with or without HDAC3 inhibition. Statistical analysis of the expression of IL-6 (i), iNOS (j), Arginase-1 (k) and RELMα (l) are shown ($n \geq 3$ biologically independent samples). Representative flow cytometry plots with HDAC3 inhibition are presented in Supplementary Fig. 18. **m**, Illustration of macrophages' type I and type II immune responses regulated by AGE-crosslinked collagen matrix. Statistical analysis was performed using one-way ANOVA with Turkey's test. Results are presented as mean $\pm$ s.e.m.

oxide synthase, encoded by *Nos2*) expression compared with those in the non-crosslinked group (Fig. 6e,f,i,j). Correspondingly, we observed that macrophages from AGE-crosslinked matrix had elevated type II

immune response, as indicated by a higher proportion of cells with upregulated Arginase-1 and RELMα expression compared with that in the non-crosslinked group (Fig. 6g,h,k,l). Furthermore, type I and

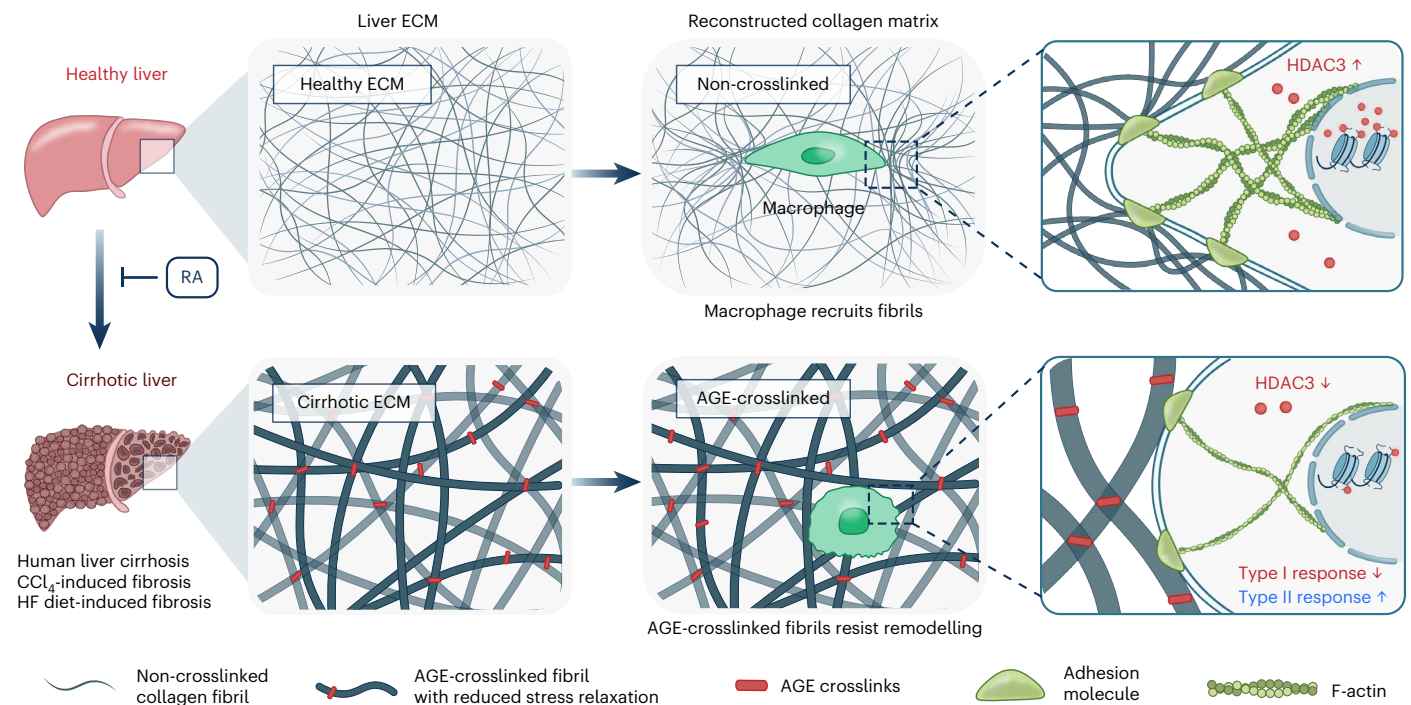


Fig. 7 | Schematic of AGE crosslinking effect on liver ECM during fibrosis progression. The high degree of AGE crosslinking in liver ECM was identified as a hallmark of liver cirrhosis, which was quantitatively verified in clinical samples with liver cirrhosis and two different animal models of liver fibrosis. The cirrhotic liver ECM showed increased elastic modulus and the collagen fibrils tightly tangled together, forming thick fibril bundles. In vitro reconstruction of AGE-crosslinked collagen matrix recapitulated the essential characteristics of in vivo liver ECM at late-fibrotic stage, as reflected in comparable AGE crosslinking

degree and similar biomechanics. The AGE-crosslinked fibrils showed decreased stress relaxation rate and therefore resisted macrophage-mediated remodeling. The fewer AGE-crosslinked fibrils being recruited to macrophages resulted in downregulated protrusion structure, adhesion molecules, cytoskeleton organization and HDAC3 expression in macrophages. Such pathological mechanotransduction resulted in downregulated type I immune response and upregulated type II immune response in macrophages, which could potentially contribute to fibrosis exacerbation.

type II immune response patterns in the AGE + RA group were similar to those in the non-crosslinked group. To explore potential crosstalk between macrophages and fibroblast activation during fibrogenesis, we performed fibroblast-embedded collagen contraction assays and found that the conditioned medium secreted by macrophages grown on AGE-crosslinked matrix induced higher fibroblast contraction (Supplementary Fig. 17).

To further investigate whether the bias in type I/type II immune response was associated with AGE-crosslinking-induced downregulation of HDAC3, we treated macrophages grown on different matrices with an HDAC3-specific inhibitor. Flow cytometry analysis showed that HDAC3 inhibition led to attenuation of both downregulation of markers related to type I immune response (that is, IL-6+ and iNOS+ proportions) and upregulation of markers related to type II immune response (that is, Arginase1+ and RELM α + proportions) in macrophages grown on AGE-crosslinked matrix. No significant differences were detected in type I/type II-related marker expression among different groups treated with HDAC3 inhibitor (Fig. 6i–l and Supplementary Fig. 18).

These results indicate that AGE-crosslinked ECM induced regulatory effects on type I/type II immune responses in macrophages via collagen fibril-mediated mechanosensing. AGE-crosslinked collagen fibrils aggregated into thick bundles with decreased stress relaxation rates and showed resistance to macrophage-mediated fibril recruitment. Therefore, decreased cell–matrix interactions result in fewer paxillin adhesions, less cytoskeletal organization and decreased HDAC3 expression in macrophages, consequently resulting in suppression of type I response and promotion of type II response, thus suggesting a pro-fibrotic role of AGE crosslinking in ECM (Fig. 7).

Rosmarinic acid inhibits AGE crosslinking in vivo

Since RA could effectively inhibit AGE crosslinking in collagen matrix in vitro, we next sought to determine whether these inhibitory effects could also be observed in vivo and possibly alleviate liver fibrosis. Mice with CCl₄-induced late-stage liver fibrosis were treated with RA or the vehicle (Fig. 8a). We then collected intact decellularized liver ECM, and SEM imaging showed that the proportion of thick fibril bundles was decreased after RA intervention. Moreover, some of the intertwined thick fibril bundles became loose, more similar to the structure of non-fibrotic liver ECM (Fig. 8b,c). The weight of decellularized ECM also decreased after RA treatment (Fig. 8d).

We quantified the degree of AGE crosslinking in liver ECM using the AQMC method and found that the total degree of AGE crosslinking was markedly lower in the RA-treated group compared with that in the vehicle-treated group; among the crosslinking structures, CML and glucosepane decreased substantially in the RA-treated group (Fig. 8e–i). Although pentosidine decreased non-significantly, its expression level remained the lowest. In addition, AGE staining confirmed that their levels were decreased in the RA-treated mice (Fig. 8j,k). The inhibitory effects of RA on AGE crosslinking in liver ECM were further confirmed in the HFDCAA diet-induced liver fibrosis model (Extended Data Fig. 6). Consistent with findings observed in CCl₄-induced models, the degree of AGE crosslinking was lower in liver ECM of RA-treated mice compared with the vehicle-treated group.

The attenuation of AGE crosslinking in liver ECM after RA intervention was accompanied by the alleviation of liver fibrosis in CCl₄-induced models, as indicated by the reduced level of collagen scar tissue, hepatic hydroxyproline and the proportion of α -SMA positive cells (Fig. 8l–n and Supplementary Fig. 19). No obvious damage was observed in organs other than the liver.

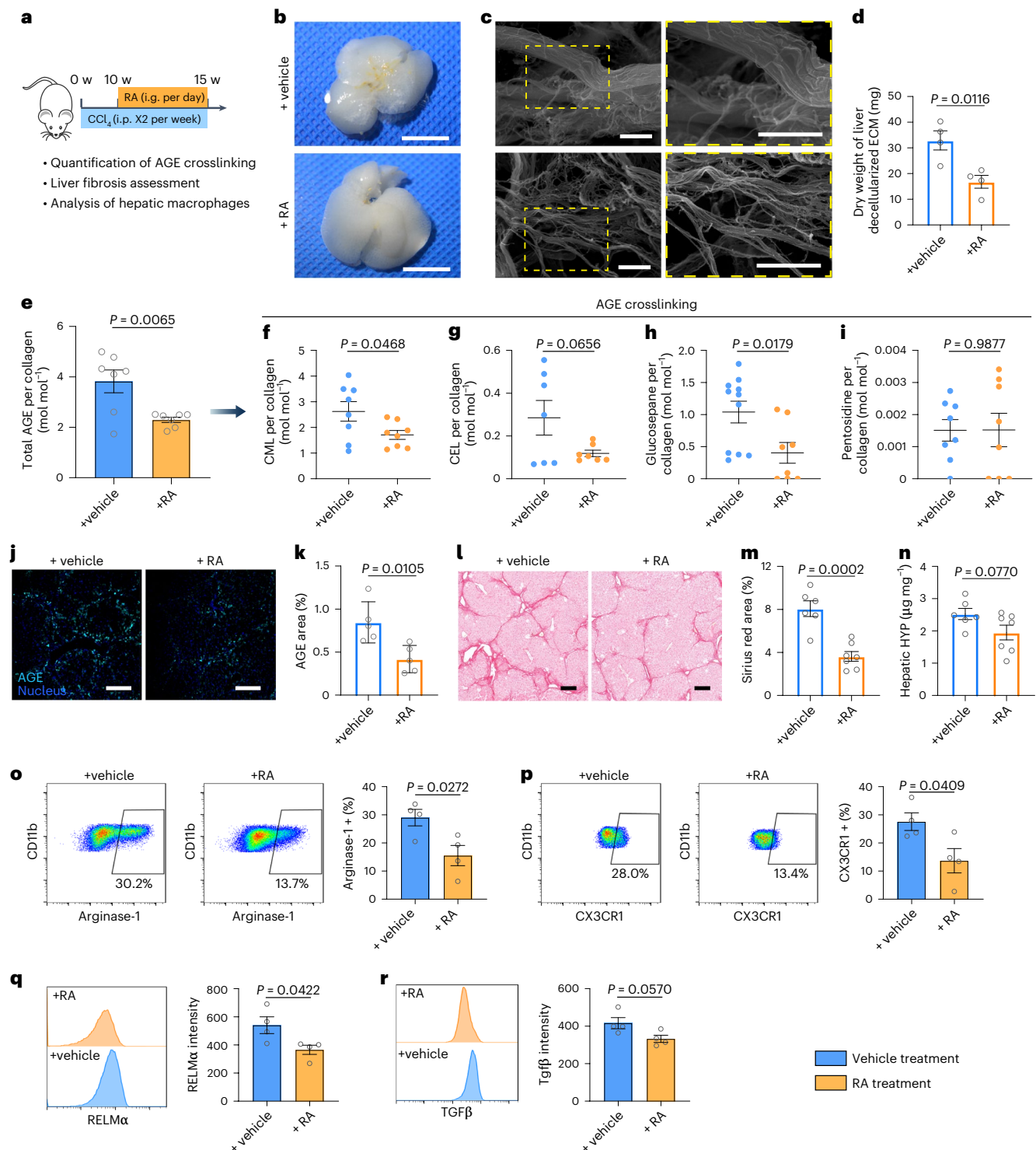


Fig. 8 | RA inhibits AGE crosslinking in liver ECM and alleviates late-stage liver fibrosis. a, Schematic of RA intervention strategy in CCl₄-induced liver fibrosis model. **b**, Representative bright-field images of liver decellularized ECM. Scale bars, 1 cm. **c**, Representative SEM images of liver decellularized ECM. Scale bars, 2 μ m. **d**, Statistical analysis of the dry weight of liver decellularized ECM obtained from whole livers (n = 4 entire liver decellularized ECMs derived from at least 4 mice per group). **e–i**, Quantification of AGE crosslinking degree in liver decellularized ECM. **e**, Total AGE crosslinking degree (n = 7); **f**, CML (n = 8); **g**, CEL (n = 7); **h**, glucosepane (n $\geq$ 8); **i**, pentosidine (n = 8). Data are presented as molar ratio of crosslinks and triple-helix tropocollagen molecules. Sample size indicates the number of pieces of liver decellularized ECM derived from at least 3 mice per group. **j,k**, Representative images (j) and statistical analysis (k) of AGE stained by immunofluorescence from livers treated with vehicle or RA (n = 5 mice

per group). Scale bars, 200 μ m. **l,m**, Representative images (l) and statistical analysis (m) of collagen (stained by Sirius red) deposition from livers treated with vehicle or RA (n $\geq$ 6 mice per group). Scale bars, 200 μ m. **n**, Quantification of hepatic hydroxyproline content. The data are expressed as hydroxyproline (μ g) per liver wet weight (mg) (n $\geq$ 6 mice per group). **o–r**, Representative flow cytometry plots (left and middle or top and bottom) and corresponding statistical analyses (right) of the expression of the type II immune response-related markers in hepatic macrophages. Gating strategy is presented in Supplementary Fig. 15. **o**, Arginase1+ populations. **p**, CX3CR1+ populations. **q**, Relative intensity of RELM α . **r**, Relative intensity of TGF β (o–r, n = 4 mice per group). Statistical analysis was performed using two-tailed unpaired t-test. Results are presented as mean $\pm$ s.e.m.

To verify whether the inhibition of AGE crosslinking could also modulate the aberrant upregulation of type II immune response of hepatic macrophages, we performed flow cytometry on hepatic macrophages isolated from mice treated with RA or the vehicle in CCl₄-induced models. The highly expressed marker related to type II immune response of hepatic macrophages (that is, Arginase1, CX3CR1 and RELM α) were downregulated after RA treatment (Fig. 8o–r).

These results thus illustrate that RA could potentially target AGE crosslinking in liver ECM and alleviate liver cirrhosis.

Discussion

We identified that a high degree of AGE crosslinking in liver ECM is a hallmark of liver cirrhosis. In vitro reconstruction of AGE-crosslinked collagen matrix helped to elucidate the effects of AGE crosslinking on collagen matrix and further revealed that AGE-crosslinked fibrils showed resistance to macrophage-mediated remodelling, resulting in an aberrant type II immune response in macrophages. Targeting AGE crosslinking could potentially alleviate liver fibrosis.

The unsatisfactory efficacy of LOX-targeted treatment in clinical trials indicates limited understanding of ECM crosslinking during fibrosis progression. Basal LOX activity is required for collagen synthesis under physiological conditions, hence targeting LOX without disrupting the homeostasis of ECM can be challenging⁴⁰. In addition, the degree of LOX crosslinking in ECM increased non-significantly compared with levels of TGM and AGE crosslinking in our fibrosis models (Fig. 2h–j) possibly because LOX crosslinking might contribute more to collagen deposition during the early stages of fibrosis than to the development of crosslinked scar tissue associated with late-stage fibrosis. Studies of diabetes and aging have also shown that LOX crosslinking does not play a dominant role in the ECM at the end stages of these chronic diseases^{41,42}.

By contrast, the degree of AGE crosslinking in the ECM remained at low levels under physiological conditions but increased to high levels in the end stages of chronic diseases after prolonged accumulation. In cirrhotic liver tissue in particular, dysregulation of glucose metabolism and insulin resistance increases the accessibility of deposited collagen to the drivers of AGE crosslinking, such as reducing sugars^{10,43}. Our quantitative results in CCl₄-induced fibrosis models show that AGE crosslinking in liver ECM undergoes the greatest fold increase during the progression of liver fibrosis. Moreover, AGE crosslinking was prevalent to a higher degree than either LOX or TGM crosslinking in clinical samples of cirrhotic liver, as well as in two different animal models. It should be noted that most patients involved in this study show abnormally high levels of fasting blood glucose (Supplementary Table 1), indicating aberrant glucose metabolism, which potentially accounts for the high degree of AGE crosslinking quantified by AQMC (Fig. 2b). These results collectively indicate that AGE crosslinking could also serve as an effective target, in addition to LOX crosslinking, to alleviate liver cirrhosis.

Although TGM-crosslinked liver ECM is also associated with liver cirrhosis³, discrepancies in the degree of TGM crosslinking were observed in different animal models (that is, upregulated in the CCl₄-induced model and downregulated in the HF-fibrosis model). Comparative analysis of clinical samples based on the disease history of patients showed that TGM crosslinking exhibited a lower degree in ECM from patients with viral hepatitis compared with that in the non-viral hepatitis group (Extended Data Fig. 7). These data suggest an aetiology-dependent feature of TGM crosslinking. In vitro reconstruction of TGM-crosslinked collagen matrix showed that TGM crosslinking had little impact on the structure of fibrils but substantially increased the elastic modulus of collagen matrix, and macrophages grown on TGM-crosslinked matrix formed protrusions (Extended Data Fig. 8). Notably, primary hepatic stellate cells (HSCs) cultured on TGM-crosslinked matrix expressed high levels of α -SMA, which represents an activated phenotype that contributes to fibrosis progression,

while upregulation of α -SMA was not observed in the AGE-crosslinked group (Supplementary Fig. 20). These results led us to conclude that TGM crosslinking in ECM could also possibly drive the progression of liver fibrosis, but in a different manner from that of AGE crosslinking.

We also characterized the structure and viscoelasticity of AGE-crosslinked collagen fibrils using Cryo-EM and optical tweezer techniques, which identified dysfunction in mechanotransduction between the ECM and cells at the single-fibril level. Optical tweezer experiments could also provide informative profiles of the viscoelasticity of cells and adjacent fibrils under physiological conditions, providing insight into the long-term interactions between macrophages and the ECM that depend on the viscoelastic properties of fibrils. Cryo-EM and 3D reconstruction of collagen fibrils together provided clues that AGE crosslinking increased both intra- and inter-fibril interactions. Further clarification of fibril structure at higher resolution could identify changes in the configuration of amino acid residues caused by crosslinking and facilitate prediction of potential targets for breaking crosslinks. In addition, analysis of possible structural changes in cell binding sites (for example, integrin-binding sites) in AGE-crosslinked fibrils could provide detailed insight into the pathogenic role of mechano-responsiveness.

Currently, there are no AGE-targeted therapeutics approved for the market⁸, and some candidates, such as aminoguanidine and alagebrium, have shown inconsistent results in animal studies and clinical trials⁴⁴. One challenge is a lack of appropriate in vitro models. AGE-crosslinked ECM has characteristics distinct from that of free AGE-crosslinked products formed in soluble proteins and lipids, which represents a major limitation for studies that use simplified BSA-AGE in vitro models^{31,45,46}. Using the AQMC method established for this work, we could effectively remove the cellular components, blood and interstitial fluid in liver by decellularization without significantly affecting the structural, physical and chemical properties of the liver ECM (Extended Data Fig. 1 and Supplementary Fig. 4). This method can thus provide major insights into the role of AGE-crosslinked ECM in chronic diseases. Moreover, quantification of the degree of crosslinking is a valuable guide for in vitro reconstruction of AGE-crosslinked ECM to accurately recapitulate the in vivo crosslinked ECM in liver cirrhosis, providing powerful tools for development of AGE-targeted strategies. Differences between the in vitro reconstructed collagen matrix and the decellularized liver ECM exist in terms of pore structure and matrix stiffness, which had non-significant effects on the cellular response of macrophages in vitro (Supplementary Figs. 21 and 22). In addition to the AGE-crosslinked collagen characterized in this study, other ECM components, such as elastin and fibronectin^{47,48}, or other types of AGE crosslinks, such as MODIC (methylglyoxal-derived imidazoline crosslink) and DODIC (3-deoxyglucosone-derived imidazoline crosslink)⁴², can also contribute to the pathogenesis of various chronic diseases. We foresee that the AQMC method can be extended to characterize more ECM components and types of ECM crosslinking, which will guide the optimization of in vitro reconstructed ECM to better recapitulate the cirrhotic liver ECM.

Our results also show that RA effectively inhibits AGE crosslinking, and its resulting effects on the biomechanics of collagen matrix both in vitro and in vivo suggest its potential for alleviating liver cirrhosis. RA treatment was previously reported to promote apoptosis in HSCs⁴⁹, inhibit inflammatory diseases⁵⁰ and alleviate early liver fibrosis^{51,52}. Although we observed decreased activation of HSCs after RA treatment, in vitro experiments indicated that RA could only deactivate HSCs at exceptionally high concentrations, suggesting that HSCs are probably not the primary targets of RA in vivo (Supplementary Fig. 23). Moreover, the highly crosslinked ECM in cirrhotic liver is quite different from the HSC-mediated collagen synthesis that occurs in early fibrosis, implying that cirrhosis patients may not benefit from targeting HSCs alone. We also found that the inhibition of AGE crosslinking in liver ECM by RA treatment during the late stages of fibrosis was accompanied by

the alleviation of type II immune responses in hepatic macrophages. However, RA treatment did not show inhibitory effects on the immune response of macrophages in vitro (Supplementary Fig. 24), indicating an indirect regulation of macrophages by RA treatment in vivo. Further investigation is needed to verify and explain the underlying mechano-regulatory effects of AGE-crosslinked ECM on hepatic macrophages in fibrotic disease. Collectively, this study suggests a potential therapeutic strategy for improving the effectiveness of liver cirrhosis treatments by targeting AGE-crosslinked scar tissue with RA.

Methods

Human liver samples

The fresh liver tissues from patients with established liver cirrhosis (patients #1–#9, as shown in Supplementary Table 1) were provided by the Department of Hepatology at Tsinghua Changgung Hospital and approved by the Institutional Review Board of Tsinghua University. Informed consent was obtained from participants. Paraffin-embedded liver tissues from healthy human (Healthy human #10, as shown in Supplementary Table 1) were purchased from OriGene Technologies. The formalin-fixed healthy liver tissue was not used in AQMC analysis and was only used for histological staining.

Animal models of liver fibrosis

C57BL/6 male mice (8-week-old) were purchased from the Laboratory Animal Resources Centre at Tsinghua University and housed in the specific-pathogen free animal facility. The mice were maintained on a 12 h/12 h light/dark cycle at 22–26 °C, with sterile pellet food and water provided ad libitum. All animal protocols used in this study were approved by the Institutional Animal Care and Use Committee of Tsinghua University. For the CCl₄-induced liver fibrosis model, 8-week-old male C57BL/6 mice received an intraperitoneal (i.p.) injection of CCl₄ (2.5 ml kg⁻¹ body weight; 1:4 diluted with olive oil) twice weekly for 4 weeks (early-stage fibrosis) or 15 weeks (late-stage fibrosis). For the HFDCAA diet-induced liver fibrosis model, 8-week-old male C57BL/6 mice were fed with the HFDCAA diet (Trophic Animal Feed High-Tech) for 21 weeks.

Liver tissue decellularization

To obtain the whole-liver decellularized ECM from mice livers, the livers were decellularized following an *in situ* perfusion protocol as illustrated in Extended Data Fig. 1b. Briefly, mouse with a certain extent of liver fibrosis was anaesthetized with 2.5% avertin, and the hepatic portal vein was cannulated with a polyethylene tube attached to a peristaltic pump. The flow rate was adjusted to 3 ml min⁻¹ and the inferior vena cava was severed to perfuse out the buffer. EGTA solutions (1.9%) were perfused for 20 min to flush out blood. Then 0.01%, 0.05% and 0.1% sodium dodecyl sulfate (SDS) solutions were sequentially perfused for 12 h in each step until the liver tissue was translucent and perfusate was negative for protein and DNA. Then deionized water was perfused for 12 h to thoroughly rinse the buffer residue in decellularized ECM. The decellularized ECM was then collected and preserved in a vacuum-drying pan after CO₂ critical-point drying. Complete decellularization was also verified by hematoxylin/eosin staining to confirm that no cellular component was left. To obtain the liver decellularized ECM from patients with liver cirrhosis, the liver tissue samples were thoroughly washed and shaken by vortex in 1% SDS buffer for 10 min, followed by sonication for 10 min. After centrifugation at 15,000 g for 15 min at 4 °C, the supernatants were discarded and the pellet was collected to repeat the process above until the supernatants were clear and white decellularized ECM with fibrous structure could be obtained from the pellet. The human liver decellularized ECMs were washed and preserved as described above. To investigate the effect of decellularization treatment on liver ECM as shown in Supplementary Fig. 4, samples were treated with 0.1% SDS for 12 h in a shaking incubator, followed by further characterization.

Sample hydrolysis

The dried samples were weighed and placed in glass hydrolysis vessels. Hydrolysis was performed using 6 N HCl. The hydrolysis vessel was sealed and incubated in a drying oven at 110 °C for 48 h. Then the samples were dried using RapidVap Vacuum Evaporation Systems (LABCONCO). For quantifying crosslinks by LC–MS/MS, the samples were dissolved in 80% methanol. For determining the hydroxyproline content, the samples were dissolved in deionized water, followed by adjustment of the pH to 7.4 using NaOH.

Isolation of crosslinking products

Samples with dry weight of 1–4 mg were used for AQMC analysis. The dried samples were weighed and transferred to a tube for enzymatic digestion. For mouse liver ECM, pieces of sample were mainly collected from the central regions of the liver lobes from the intact ECM, which showed relatively homogeneous texture. The ECM near the regions at the linkage of large blood vessels or ligaments showing high heterogeneity, such as the region near the portal vein, was excluded from further analysis. Stepwise, the following proteases were added every 24 h: (1) 2 mg ml⁻¹ collagenase I (GIBCO, 17100-017) in 1 ml TRIS-HCl buffer (pH 8); (2) 0.8 U pronase (Roche, 11459643001); (3) 0.8 U pronase and 0.5 mg papain (Sigma, P4762); (4) 0.8 U aminopeptidase M (Shanghai Yuanye, S25360), 1 U prolidase (Sigma, P6675) and 10 mM Mg²⁺/Mn²⁺ to activate the enzyme; (5) 0.8 U aminopeptidase M; and (6) 0.65 U carboxypeptidase (Sigma, SAE0046). Around 1 mg thymol (Macklin, T818893) was added to the samples to inhibit bacteria. The volume of the reaction system was kept at 1 ml and the used tubes were sealed to avoid evaporation. The samples were incubated in a shaking incubator at 37 °C. Enzyme was inactivated by vortex and sonication for 10 min at 100 °C after each step. Quality control was performed to evaluate the efficiency of enzymatic digestion by comparing the content of hydroxyproline in samples treated by enzymatic digestion and acid hydrolysis using HPLC–MS/MS. After the final step, enzyme residues were sedimented by cold methanol at –80 °C for 1 h, and were discarded after centrifugation at 13,000 g for 15 min. Around 5 ml of methanol was used for the treatment of a sample. The crosslinks in supernatants were collected and dried by a RapidVap Vacuum Evaporation System (LABCONCO). The samples were dissolved in 80 µl 80% methanol for the detection of γ-Glutamyl-ε-Lysine, glucosepane and pentosidine by HPLC–MS/MS. The samples were further hydrolysed using 6 N HCl for another 48 h as described in the sample hydrolysis part for the detection of pyridinoline, CML and CEL.

HPLC–MS/MS

The HPLC system (Japan Jasco) consisted of a five-way online degasser (ExionLc Degasser-5036645) and an ultra-efficient binary pump (ExionLc AD Pump-5036653) with a solvent mixer (20 µl Micro Mixer-45054489), column thermostat (ExionLc AD Oven-5036656) and autosampler (ExionLc AD Autosampler-5036654). Mass detection was performed on an AB SCIEX QTRAP 5500 triple quadrupole mass spectrometer equipped with a turbo ion spray source using electrospray ionization in positive ion mode. The MS parameters were as follows: ESI ion source temperature of 500 °C; air curtain of 30 psi; collision activated dissociation gas settings: medium, ion spray voltage of 5,500 V, ion gas 1 and 2 of 50 psi. Chromatographic separation was performed on a Luna NH2 analytical column (2× 100 mm, 3 µm, Phenomenex) equipped with an ExionLc AD system (AB Sciex) infinite binary pump (ExionLc AD Pump-5036653). The mobile phase included acetonitrile containing 20 mM ammonium acetate and 0.2% formic acid (eluent A) and water containing 20 mM ammonium acetate and 0.2% formic acid (eluent B). Gradient elution was performed using a flow rate of 0.4 ml min⁻¹ at a column temperature of 25 °C: 10% B for 8 min, 80% B for 2 min, 10% B for 4 min. The sample was kept at 10 °C and the injection volume was 5 µl. For mass spectrometry detection, multiple reaction

monitoring mode was used, with collision-induced dissociation of protonated molecules, compound-specific declustering potential and fragment-specific collision energy. The MS₂ fragmentation spectra of the crosslinking products used for detection are shown in Extended Data Fig. 2, and relevant information is listed in Supplementary Table 2.

Determination of crosslinking degree

MS data were analysed using AB SCIEX Analyst 1.7.1. Quantification was performed using external standard methods. Briefly, standard crosslinking compound mixtures of increasing concentrations were analysed by HPLC–MS/MS using the same methods as described above. A regression of response versus concentration of standard was used to determine the concentration of the corresponding crosslinking products in the sample. Background signals caused by the isolation and preparation process were deducted before determining the content of crosslinking products. The crosslinking degree mediated by a specific crosslinking mechanism in a sample was defined as the molar ratio (mole mole^{−1}) of crosslinking products to the tropocollagen molecules detected in the sample, as illustrated in Extended Data Fig. 1. It should be noted that the quantification results obtained from healthy ECM samples in the HF-fibrosis model shown in Extended Data Fig. 3 are the same as those in the CCl₄-induced model shown in Fig. 2 since 8-week-old untreated mice were used as healthy control for both liver fibrosis models in AQMC assays. The degrees of LOX and TGM crosslinking were determined by pyridinoline and γ-Glutamyl-ε-Lysine, respectively. The total degree of AGE crosslinking was determined by the molar sum of CML, CEL, glucosepane and pentosidine. The mole quantity of triple-helix tropocollagen molecules was determined by hydroxyproline (300 hydroxyproline residues per tropocollagen molecule⁵³). Authentic standards used for determination include pyridinoline (Toronto Research, H954036), γ-Glutamyl-ε-Lysine (Sigma, G5136), N^ε-(1-carboxymethyl)-L-lysine (CML, Cayman Chemical, 16483), N^ε-(1-carboxyethyl)-L-lysine (CEL, Cayman Chemical, 25333), pentosidine (Cayman Chemical, 10010254) and hydroxyproline (Solarbio, SH8250).

Optical tweezer measurements

Viscoelasticity of the bulk collagen matrix was characterized using an mmi CellManipulator optical trapping system (mmi Cellmanipulator, MM1) following a modified protocol⁵⁴ as illustrated in Fig. 4a. Uncoated polystyrene beads ($d = 5 \mu\text{m}$, hugebio) were embedded in collagen matrix during the preparation and manipulated by an optical trap. The laser beam (8 W, 1,070 nm) was tightly focused through a series of Keplerian beam expanders and a high-NA objective ($\times 60$ 1.2 NA; water; Nikon). A high-resolution quadrant detector was used for position detection. Calibration of the linear region of the detector and the trap relative elastic modulus (60 pN nm^{−1}) was performed in PBS using the same type of beads according to the harmonic oscillation method. After a bead was trapped by the laser, the trapping laser was moved along a certain direction at a constant velocity of $1 \mu\text{m s}^{-1}$ to manipulate the bead. The force-displacement curve was recorded. The matrix stiffness of the collagen matrix was determined by the slope of the linear region of the force-displacement curve. Since the dimensional parameters were identical across different groups, that is, the diameter of the bead and the dimension of the collagen matrix, the matrix stiffness was linearly correlated with the Young's modulus of the collagen matrix. To determine the stress relaxation rate of the collagen matrix, the bead was manipulated by an optical trap as described above until the detected force reached 30 pN. Then the bead was kept static while the load was recorded as a function of time. The stress relaxation data of the collagen matrix were fit to a two-element Maxwell–Weichert linear viscoelastic model with 2 Maxwell elements in parallel⁵⁵. The time for the initial stress to be relaxed to half its value during a stress relaxation test was defined as the half stress relaxation time ($\tau^{1/2}$).

To simulate the cell-traction force to remodel the collagen fibrils, polystyrene beads ($d = 5 \mu\text{m}$, hugebio) were embedded in collagen

matrix and assay was performed in the mmi CellManipulator optical trapping system as illustrated in Fig. 5c. A constant force of 50 pN comparable to the cell-traction force generated by macrophages was applied by an optical trap to induce the displacement of the bead. The gradual displacements of the surrounding collagen fibrils caused by the bead were recorded by time-lapse imaging during a period of 30 s. The displacements of fibrils were analysed using Imaris.

To characterize the viscoelasticity of individual collagen fibrils, a dual-trap optical tweezer instrument with confocal fluorescence imaging system (C-trap; Lumicks) was used. Biotin moieties were covalently linked to the N terminus of collagen I monomers utilizing the cysteine residues following a modified protocol as described previously^{56,57}. Briefly, 5 mM tris(2-carboxyethyl)phosphine of a final concentration (Thermo Fisher, 77720) was added to 0.5 mg ml^{-1} collagen solutions, followed by incubation at 37 °C for 0.5 h. Biotins were covalently linked to collagen monomers by adding $0.04 \mu\text{M}$ maleimide-biotin (EZ-Link Maleimide-PEG2-Biotin, Thermo Fisher) to the reacting solutions for another incubation time of 12 h at 4 °C. Biotinylated collagen was purified by dialysis for 24 h. The collagen solution was diluted by assembling buffer to a final concentration of 0.2 mg ml^{-1} and incubated at 37 °C for 96 h in a humidified atmosphere to obtain the individual collagen fibrils. Collagen solutions were supplemented with additional glucose and RA to obtain AGE-crosslinked collagen fibrils and AGE + RA fibrils. Two streptavidin-coated beads ($d = 4.45 \mu\text{m}$, Spherotech) were manipulated by two optical traps (trap A, mobile trap; trap B, stable trap), each bound to one biotinylated site within a single collagen fibril. Axial stretching of collagen fibrils was performed by moving trap A along the axial direction of collagen fibrils at a velocity of $1 \mu\text{m s}^{-1}$. Trap stiffness, representing the spring constant of the optical trap, was obtained by fitting a power spectrum of the same trapped bead to an active power-spectrum method and equipartition theorem. The trap stiffness for the measurements was $200 \text{ pN } \mu\text{m}^{-1}$. Forces on the trapped beads were determined using the trap stiffness and bead offset from equilibrium. Trap displacement was determined from the separation between beads and included an additional distance offset term when fitting the data to account for potential off-axis tethering. We fitted force-displacement curves using the modified extensible worm-like-chain model (WLC)⁵⁸ as in equation (1):

$$F = \left(\frac{k_B T}{L_p} \right) \left[\frac{1}{4(1-x/L_0+F/K)^2} - \frac{1}{4} + \frac{x}{L_0} - \frac{F}{K} \right] \quad (1)$$

where F is the extension of the fibril (end-to-end distance), k_B is Boltzmann's constant, T is the absolute temperature, x is the displacement of bead, L_0 is the initial length of the fibril, L_p is the persistence length of the fibril and K is the material parameter representing enthalpic compliance of the fibril. Only data that fitted well with the WLC model was considered to be obtained from a single fibril and was used for subsequent analysis. Young's modulus of a single collagen fibril was determined by fitting the linear region of the stress-strain curve.

To determine the stress relaxation rate of collagen fibrils, one of the beads binding to one end of the collagen fibril was manipulated by the mobile optical trap to stretch the fibril along the axial direction. When the strain of the collagen fibril reached 7%, the bead was kept static while the load was recorded as a function of time. The stress relaxation data of the collagen fibril were fitted to a two-element Maxwell–Weichert linear viscoelastic model with 2 Maxwell elements in parallel. The time for the initial stress to be relaxed to half its value during a stress relaxation test was defined as the half stress relaxation time ($\tau^{1/2}$). To verify the stability of manipulating the system, the biotinylated double-stranded λ -DNA (48,524 bp, Lumicks) was selected as an elastic standard to detect stress relaxation in the same way as detecting that of a single collagen fibril. No significant decrease in stress was detected, indicating that the detected decreases in stress were specifically caused by the stress relaxation of samples, as shown in Fig. 4g.

BMDM isolation

Mouse BMDM were prepared from healthy C57BL/6 male mice (8–12 weeks old) as described previously³⁹. In brief, bone marrow from femurs and tibiae was collected and filtered through a 40 µm cell strainer to remove debris. The cells were resuspended in DMEM medium containing 10% heat-inactivated FBS, 1% penicillin–streptomycin solution (Gibco) and 15% L929-conditioned medium, and distributed into 100 mm Petri-dishes. The cells were cultured for 7 d and yielded a macrophage (CD11b⁺ F4/80⁺) population of up to 95% as assessed by flow cytometry.

Study of BMDM grown on crosslinked collagen matrix in vitro

The prepared crosslinked collagen matrix was thoroughly washed with PBS to remove reagent residues. Quality control was performed to confirm that reagent residues, especially endotoxins, were at very low level and could not induce unexpected response of macrophages as described in Extended Data Fig. 4. BMDM were collected and suspended in DMEM medium containing 10% FBS and 1% penicillin–streptomycin solution. Cell suspensions were applied into the collagen matrix at a concentration of 7.5×10^5 cells per ml. The collagen matrix was transferred into a cell culture incubator and incubated for 36 h without disturbance to allow the cells to adhere to the collagen matrix. The medium was then discarded, followed by careful supplementation with sufficient fresh medium. Then the cells were grown in the collagen matrix for 96 h. To assess the type I response of macrophages, 20 ng ml⁻¹ LPS (Sigma L3129) was introduced into the culture medium. Macrophage RNA was isolated 6 h after induction and macrophages were collected 12 h after induction as illustrated in Fig. 6a. To assess the type II response of macrophages, 20 ng ml⁻¹ IL-4 (PeproTech) was introduced into the culture medium. RNA and cells were isolated 24 h after induction as illustrated in Fig. 6c. To perform intracellular staining assay by flow cytometry, protein transport was inhibited using GolgiStop (BD, 554724) in the last 10 h before collecting cells according to manufacturer instructions. For RGD pre-treatment, cells were incubated with 0.2 mg ml⁻¹ RGD (Abcam, 142689) for 30 min at 4 °C, followed by seeding into the prepared collagen matrix. For applying increased osmotic pressure to cells, 400 Da polyethylene glycol (PEG-400, Sigma) at a final concentration of 6% (v/v) was introduced into the culture medium and the cells were treated with the increased osmotic pressure for 24 h, as described previously^{38,39}. For HDAC3 inhibition, cells were treated with 2 µM RGFP966 (Selleckchem, S7229) for 24 h before adding LPS or IL-4, and RGFP966 was kept in the medium during the subsequent process. All the reagents were verified to be endotoxin free (below 0.01 EU ml⁻¹). Endotoxin residues in reagents were removed using ToxinEraser endotoxin removal kit (Genscript, L00338). Endotoxin residues were tested using ToxinSensor Chromogenic LAL endotoxin assay kit (Genscript, L00350C). Glucose residues were determined with a Sinocare automatic glucometer and RA residues were determined by LC–MS/MS.

RNA isolation and real-time qPCR analysis

Total RNA extraction reagent (Vazyme) was used to isolate RNA. For isolating RNA from cells grown in collagen matrix, cell-embedded collagen matrix was treated with total RNA extraction reagent with additional vortexing for 20 min. Complementary DNA was prepared using a Hiscript II qRT SuperMix kit (V) (Vazyme). Real-time qPCR experiments were performed using AceQ qPCR SYBR Green master mix (Vazyme) in a CFX96 Real-Time PCR Detection System (Bio-Rad). The primer and their corresponding sequences used for real-time qPCR analysis are given in Supplementary Table 3, with *18S rRNA* and *GAPDH* as housekeeping genes for mouse and human-derived cells (LX-2), respectively.

Flow cytometry

For primary hepatic macrophage analysis, hepatic macrophages were preliminarily collected and purified from liver tissues as described in

Primary cells isolation in the supplementary methods. For analysing BMDM growing in collagen matrix, cells were collected by degrading the collagen matrix with collagenase type I (5 mg ml⁻¹, GIBCO 17100-017) at 37 °C for 30 min. Collected cells were washed, fixed and blocked using Cytofix/Cytoperm kit (BD Biosciences, 554724), followed by blocking with TruStain FcX PLUS (Biolegend, 156603). Cells were stained with fluorescence conjugated antibodies at 4 °C for 30 min. The gating strategies for identification of hepatic macrophages (CD45⁺ Ly6G[–] CD11b⁺ F4/80⁺) and BMDM (CD11b⁺ F4/80⁺) are shown in Supplementary Figs. 15b and 18a, respectively. The following antibodies were used: CD45 (Biolegend, 103155), Ly6G (Biolegend, 127626), CD11b (Biolegend, 101251), F4/80 (Biolegend, 123114), Arginase1 (eBioscience, 17-3697-82), RELMα (eBioscience, 12-5441-80), iNOS (eBioscience, 17-5920-80), IL-6 (Biolegend, 504504), CX3CR1 (Biolegend, 149005) and Tgfβ (Biolegend, 141405). Flow cytometry was performed using a BD LSRFortessa SORP. All data were analysed using FlowJo v10.

Immunofluorescence staining

Cells growing on collagen matrix were fixed with 4% paraformaldehyde in microtubule-stabilizing buffer (100 mM PIPES, 5 mM EGTA, 2 mM MgCl₂ (pH 6.8)) for 30 min as described previously³⁶ and washed with PBS three times. Cells were permeabilized and blocked with PBS containing 0.3% Triton X-100 (Sigma) and 3% bovine serum albumin (BSA) (Amresco) for 1 h with gentle shaking. Cells were then incubated with primary antibodies (diluted in 1% BSA) at 4 °C overnight, followed by washing three times with PBS. The primary antibodies used were anti-paxillin (Abcam, ab32115) and anti-HDAC3 (Abcam, ab32369). Then the cells were incubated with corresponding secondary antibodies for 1 h. Nuclei were stained with Hoechst 33342 (Beyotime, 1:2,000) for 15 min. Filamentous actin (F-actin) was labelled using Acti-stain 488 phalloidin or Acti-stain 555 phalloidin (1:200; Cytoskeleton) and globular actin (G-actin) was labelled using DNase-I AlexaFluor 488 conjugate (1:500; D12371, Life Technologies). All the antibodies used in this study are listed in Supplementary Table 4.

Statistics and reproducibility

Statistical analyses were performed using GraphPad Prism v8.4.2. Data are presented as mean ± s.d. or s.e.m. as indicated in figure legends. Statistical significance was determined using two-tailed unpaired Student's *t*-test for comparison of two groups or by ANOVA for comparison of multiple groups. Exact *P* values are marked in the figures. Representative micrographs from at least three biological replicates are shown. Details of sample sizes and statistical tests for experiments are specified in the figure legends.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The main data supporting the results in this study are available within the paper and its Supplementary Information. The raw RNA-seq data are available at the Sequence Read Archive database via the accession numbers [PRJNA852213](#) and [PRJNA856261](#). Source data are provided with this paper.

References

- Schuppan, D. & Afdhal, N. H. Liver cirrhosis. *Lancet* **371**, 838–851 (2008).
- Tsochatzis, E. A., Bosch, J. & Burroughs, A. K. Liver cirrhosis. *Lancet* **383**, 1749–1761 (2014).
- Issa, R. et al. Spontaneous recovery from micronodular cirrhosis: evidence for incomplete resolution associated with matrix cross-linking. *Gastroenterology* **126**, 1795–1808 (2004).

4. Liu, S. B. et al. Lysyl oxidase activity contributes to collagen stabilization during liver fibrosis progression and limits spontaneous fibrosis reversal in mice. *FASEB J.* **30**, 1599–1609 (2016).
5. Sorushanova, A. et al. The collagen suprafamily: from biosynthesis to advanced biomaterial development. *Adv. Mater.* **31**, e1801651 (2019).
6. Harrison, S. A. et al. Simtuzumab is ineffective for patients with bridging fibrosis or compensated cirrhosis caused by nonalcoholic steatohepatitis. *Gastroenterology* **155**, 1140–1153 (2018).
7. Fickert, P. Is this the last requiem for simtuzumab? *Hepatology* **69**, 476–479 (2019).
8. Lampi, M. C. & Reinhart-King, C. A. Targeting extracellular matrix stiffness to attenuate disease: from molecular mechanisms to clinical trials. *Sci. Transl. Med.* **10**, eaao0475 (2018).
9. Monnier, V. M. et al. Cross-linking of the extracellular matrix by the Maillard reaction in aging and diabetes: an update on ‘a puzzle nearing resolution’. *Ann. N. Y. Acad. Sci.* **1043**, 533–544 (2005).
10. Garcia-Compean, D. et al. The prevalence and clinical characteristics of glucose metabolism disorders in patients with liver cirrhosis. A prospective study. *Ann. Hepatol.* **11**, 240–248 (2012).
11. Guo, C. H. et al. The investigation of glucose metabolism and insulin secretion in subjects of chronic hepatitis B with cirrhosis. *Int. J. Clin. Exp. Pathol.* **8**, 13381–13386 (2015).
12. Elkrif, L. et al. Diabetes mellitus in patients with cirrhosis: clinical implications and management. *Liver Int.* **36**, 936–948 (2016).
13. Nkontchou, G. et al. Insulin resistance, serum leptin, and adiponectin levels and outcomes of viral hepatitis C cirrhosis. *J. Hepatol.* **53**, 827–833 (2010).
14. Brings, S. et al. Dicarbonyls and advanced glycation end-products in the development of diabetic complications and targets for intervention. *Int. J. Mol. Sci.* **18**, 984 (2017).
15. Snedeker, J. G. & Gautieri, A. The role of collagen crosslinks in ageing and diabetes - the good, the bad, and the ugly. *Muscles* **4**, 303–308 (2014).
16. Henning, C. & Glomb, M. A. Pathways of the Maillard reaction under physiological conditions. *Glycoconj. J.* **33**, 499–512 (2016).
17. Fessel, G. et al. Advanced glycation end-products reduce collagen molecular sliding to affect collagen fibril damage mechanisms but not stiffness. *PLoS ONE* **9**, e110948 (2014).
18. Hall, M. S. et al. Fibrous nonlinear elasticity enables positive mechanical feedback between cells and ECMs. *Proc. Natl Acad. Sci. USA* **113**, 14043–14048 (2016).
19. Maller, O. et al. Tumour-associated macrophages drive stromal cell-dependent collagen crosslinking and stiffening to promote breast cancer aggression. *Nat. Mater.* **20**, 548–559 (2021).
20. Baker, B. M. et al. Cell-mediated fibre recruitment drives extracellular matrix mechanosensing in engineered fibrillar microenvironments. *Nat. Mater.* **14**, 1262–1268 (2015).
21. Liu, L. W. et al. Matrix-transmitted paratensile signaling enables myofibroblast-fibroblast cross talk in fibrosis expansion. *Proc. Natl Acad. Sci. USA* **117**, 10832–10838 (2020).
22. Ramachandran, P. & Iredale, J. P. Macrophages: central regulators of hepatic fibrogenesis and fibrosis resolution. *J. Hepatol.* **56**, 1417–1419 (2012).
23. Klepfish, M. et al. LOXL2 inhibition paves the way for macrophage-mediated collagen degradation in liver fibrosis. *Front. Immunol.* **11**, 480 (2020).
24. Van Goethem, E., Poincloux, R., Gauffre, F., Maridonnoeu-Parini, I. & Le Cabec, V. Matrix architecture dictates three-dimensional migration modes of human macrophages: differential involvement of proteases and podosome-like structures. *J. Immunol.* **184**, 1049–1061 (2010).
25. Wynn, T. A. Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat. Rev. Immunol.* **4**, 583–594 (2004).
26. Gordon, S. & Martinez, F. O. Alternative activation of macrophages: mechanism and functions. *Immunity* **32**, 593–604 (2010).
27. Lopez-Navarrete, G. et al. Th2-associated alternative Kupffer cell activation promotes liver fibrosis without inducing local inflammation. *Int. J. Biol. Sci.* **7**, 1273–1286 (2011).
28. Garcia-Tsao, G., Friedman, S., Iredale, J. & Pinzani, M. Now there are many (stages) where before there was one: in search of a pathophysiological classification of cirrhosis. *Hepatology* **51**, 1445–1449 (2010).
29. Wei, G. et al. Comparison of murine steatohepatitis models identifies a dietary intervention with robust fibrosis, ductular reaction, and rapid progression to cirrhosis and cancer. *Am. J. Physiol. Gastrointest. Liver Physiol.* **318**, G174–G188 (2020).
30. Hu, M. et al. Hepatic macrophages act as a central hub for relaxin-mediated alleviation of liver fibrosis. *Nat. Nanotechnol.* **16**, 466–477 (2021).
31. Jean, D., Pouligon, M. & Dalle, C. Evaluation in vitro of AGE-crosslinks breaking ability of rosmarinic acid. *Glycative Stress Res.* **2**, 204–207 (2015).
32. Ou, J. Y., Huang, J. Q., Wang, M. F. & Ou, S. Y. Effect of rosmarinic acid and carnosic acid on AGEs formation in vitro. *Food Chem.* **221**, 1057–1061 (2017).
33. Gautieri, A., Redaelli, A., Buehler, M. J. & Vesentini, S. Age- and diabetes-related nonenzymatic crosslinks in collagen fibrils: candidate amino acids involved in Advanced Glycation End-products. *Matrix Biol.* **34**, 89–95 (2014).
34. Cao, X. et al. Multiscale model predicts increasing focal adhesion size with decreasing stiffness in fibrous matrices. *Proc. Natl Acad. Sci. USA* **114**, E4549–E4555 (2017).
35. Adebowale, K. et al. Enhanced substrate stress relaxation promotes filopodia-mediated cell migration. *Nat. Mater.* **20**, 1290–1299 (2021).
36. Jain, N. & Vogel, V. Spatial confinement downsizes the inflammatory response of macrophages. *Nat. Mater.* **17**, 1134–1144 (2018).
37. Nguyen, H. C. B., Adlanmerini, M., Hauck, A. K. & Lazar, M. A. Dichotomous engagement of HDAC3 activity governs inflammatory responses. *Nature* **584**, 286–290 (2020).
38. Lee, H. P., Gu, L., Mooney, D. J., Levenston, M. E. & Chaudhuri, O. Mechanical confinement regulates cartilage matrix formation by chondrocytes. *Nat. Mater.* **16**, 1243–1251 (2017).
39. Jiang, S. et al. Cryoprotectant enables structural control of porous scaffolds for exploration of cellular mechano-responsiveness in 3D. *Nat. Commun.* **10**, 3491 (2019).
40. Kagan, H. M. & Li, W. D. Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. *J. Cell. Biochem.* **88**, 660–672 (2003).
41. Stammers, M. et al. Age-related changes in the physical properties, cross-linking, and glycation of collagen from mouse tail tendon. *J. Biol. Chem.* **295**, 10562–10571 (2020).
42. Jost, T., Zipprich, A. & Glomb, M. A. Analysis of advanced glycation endproducts in rat tail collagen and correlation to tendon stiffening. *J. Agric. Food Chem.* **66**, 3957–3965 (2018).
43. Petrides, A. S., Vogt, C., Schulzeberge, D., Matthews, D. & Strohmeyer, G. Pathogenesis of glucose-intolerance and diabetes mellitus in cirrhosis. *Hepatology* **19**, 616–627 (1994).
44. Nenna, A. et al. Basic and clinical research against Advanced Glycation End Products (AGEs): new compounds to tackle cardiovascular disease and diabetic complications. *Recent Adv. Cardiovasc. Drug Discov.* **10**, 10–33 (2015).

45. Goodwin, M. et al. Advanced glycation end products augment experimental hepatic fibrosis. *J. Gastroenterol. Hepatol.* **28**, 369–376 (2013).
46. Valencia, J. V., Mone, M., Koehne, C., Rediske, J. & Hughes, T. E. Binding of receptor for advanced glycation end products (RAGE) ligands is not sufficient to induce inflammatory signals: lack of activity of endotoxin-free albumin-derived advanced glycation end products. *Diabetologia* **47**, 844–852 (2004).
47. Gaar, J., Naffa, R. & Brimble, M. Enzymatic and non-enzymatic crosslinks found in collagen and elastin and their chemical synthesis. *Org. Chem. Front.* **7**, 2789–2814 (2020).
48. Kong, W. Y., Lyu, C., Liao, H. G. & Du, Y. N. Collagen crosslinking: effect on structure, mechanics and fibrosis progression. *Biomed. Mater.* <https://iopscience.iop.org/article/10.1088/1748-605X/ac2b79> (2021).
49. Zhang, J. J., Wang, Y. L., Feng, X. B., Song, X. D. & Lu, W. B. Rosmarinic acid inhibits proliferation and induces apoptosis of hepatic stellate cells. *Biol. Pharm. Bull.* **34**, 343–348 (2011).
50. Luo, C. et al. A review of the anti-inflammatory effects of rosmarinic acid on inflammatory diseases. *Front. Pharm.* **11**, 153 (2020).
51. Yang, M. D. et al. Rosmarinic acid and baicalin epigenetically derepress peroxisomal proliferator-activated receptor gamma in hepatic stellate cells for their antifibrotic effect. *Hepatology* **55**, 1271–1281 (2012).
52. Domitrovic, R. et al. Rosmarinic acid ameliorates acute liver damage and fibrogenesis in carbon tetrachloride-intoxicated mice. *Food Chem. Toxicol.* **51**, 370–378 (2013).
53. Levental, K. R. et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell* **139**, 891–906 (2009).
54. Han, Y. L. et al. Cell contraction induces long-ranged stress stiffening in the extracellular matrix. *Proc. Natl Acad. Sci. USA* **115**, 4075–4080 (2018).
55. Chaudhuri, O. et al. Hydrogels with tunable stress relaxation regulate stem cell fate and activity. *Nat. Mater.* **15**, 326–334 (2016).
56. Rezaei, N. et al. Using optical tweezers to study mechanical properties of collagen. *Proc. SPIE* <https://doi.org/10.1117/12.905714> (2011).
57. Jiao, J. Y., Rebane, A. A., Ma, L. & Zhang, Y. L. Single-molecule protein folding experiments using high-precision optical tweezers. *Methods Mol. Biol.* **1486**, 357–390 (2017).
58. Wang, M. D., Yin, H., Landick, R., Gelles, J. & Block, S. M. Stretching DNA with optical tweezers. *Biophys. J.* **72**, 1335–1346 (1997).

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Author contributions

C.L. and Y.D. conceived and designed the research. C.L. and W.K. performed the AQMC assays, collagen matrix construction, BMDM studies and animal experiments. Z.L. performed the optical tweezer experiments. W.K. and S.W. performed the cryo-EM experiments and established 3D collagen fibril models with the guidance of X.L. W.Y. and C.X. provided the clinical tissue samples and helped with the clinical consultation. P.Z. helped with the animal studies. K.L. prepared the illustrations. Y.N. helped with the preparation of stiffened collagen matrix. X.H. provided the guidance for the BMDM studies. C.L. and Y.D. wrote the paper. Y.D. is the principal investigator of the supporting grants.

Competing interests

The authors declare no competing interests.

Additional information

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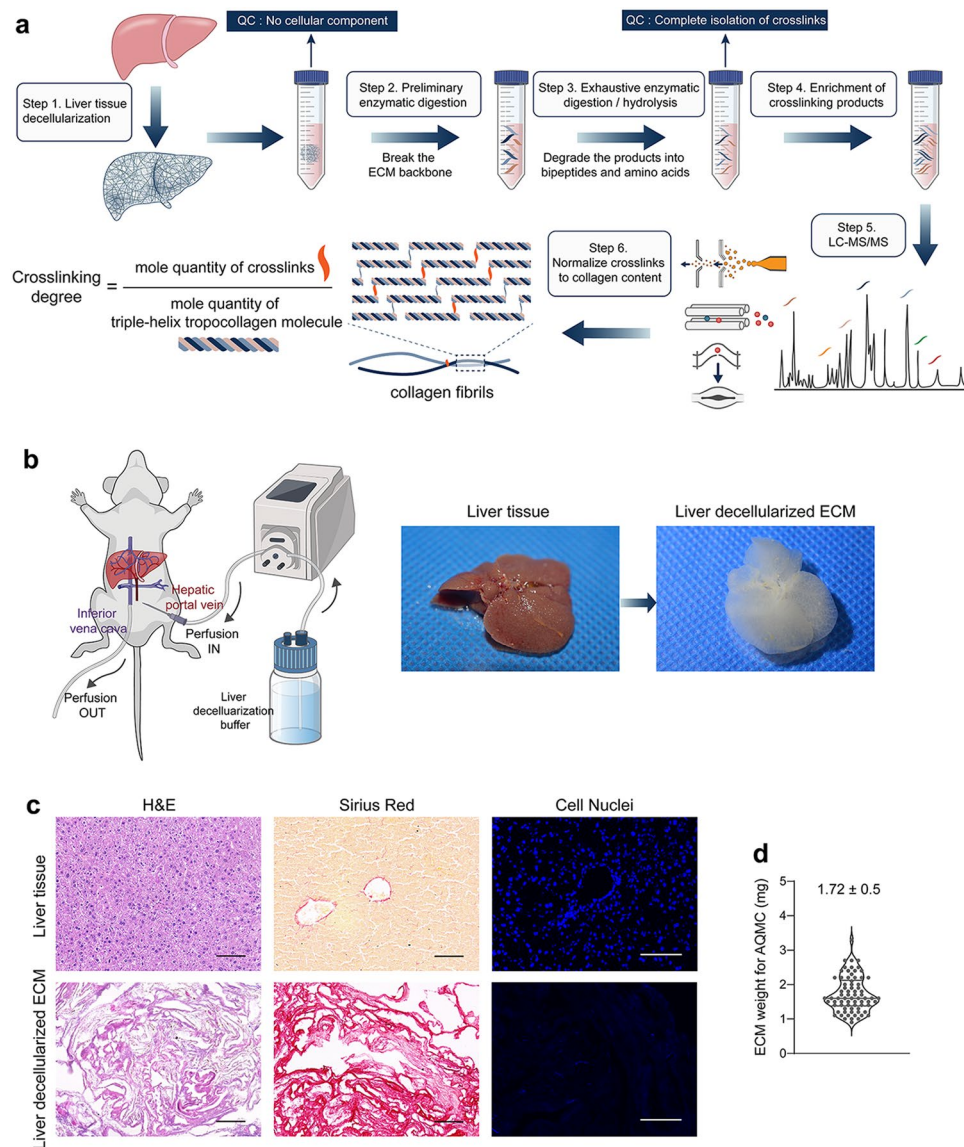
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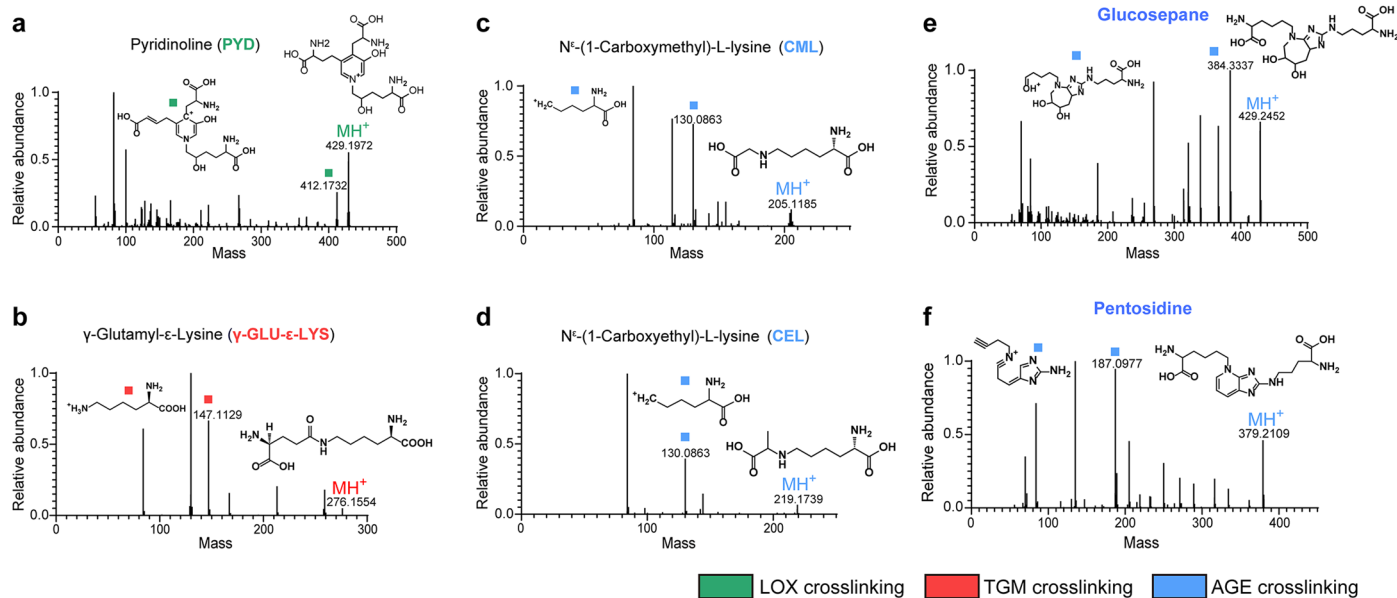
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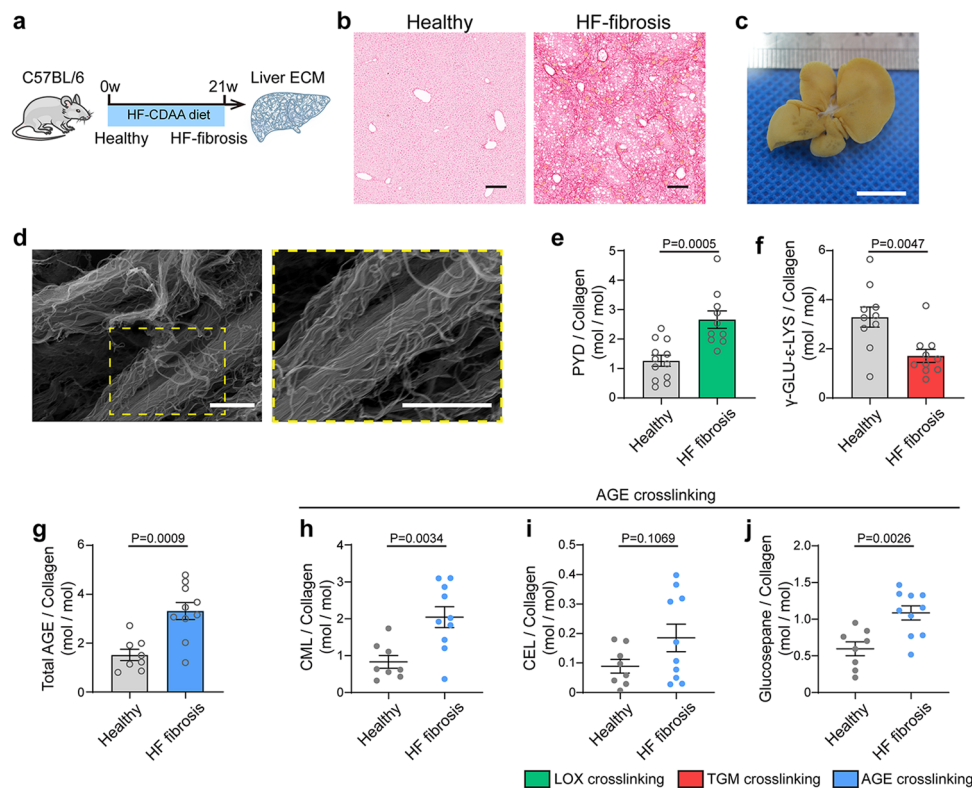
Extended Data Fig. 1 | Schematic of developing Absolute Quantification of Matrix-specific Crosslinking (AQMC) method. **a**, Flow chart of quantifying crosslinking degree of liver decellularized ECM using AQMC method (detailed in *Methods* section). (1) Harvesting the liver decellularized ECM. Quality control is performed to verify the complete de-cell process without cellular component left. (2) Degradation of the liver ECM into fragments. (3) Complete degradation of the fragments into dipeptides and amino acids. (4) Enrichment of the crosslinking products. (5) Absolute quantification of the mole quantity of crosslinking products and hydroxyproline using LC-MS/MS. (6) Calculation of the crosslinking degree. The crosslinking degree of a certain type of crosslinking is defined as the molar ratio of crosslinking products and tropocollagen molecules.

The mole quantity of triple-helix tropocollagen molecules is calculated according to the content of hydroxyproline as detailed in *Methods* section. **b**, Schematic of preparing mouse liver decellularized ECM using the whole-liver-perfusion method. The decellularization buffer is perfused into the liver from the hepatic portal vein and effused out from the inferior vena cava, as detailed in *Methods* section. **c**, Quality control of the mouse liver decellularization ECM performed by HE, Sirius Red, and cell nuclei staining. Nuclear components in liver tissue were removed by decellularization, whereas main ECM components (that is, collagen fibrils) were preserved as indicated by Sirius Red staining. Scale bars, 100 μm . **d**, Dry weight of liver decellularized ECM samples used for AQMC test. The mean $\pm$ s.e.m. values are marked on the plot.



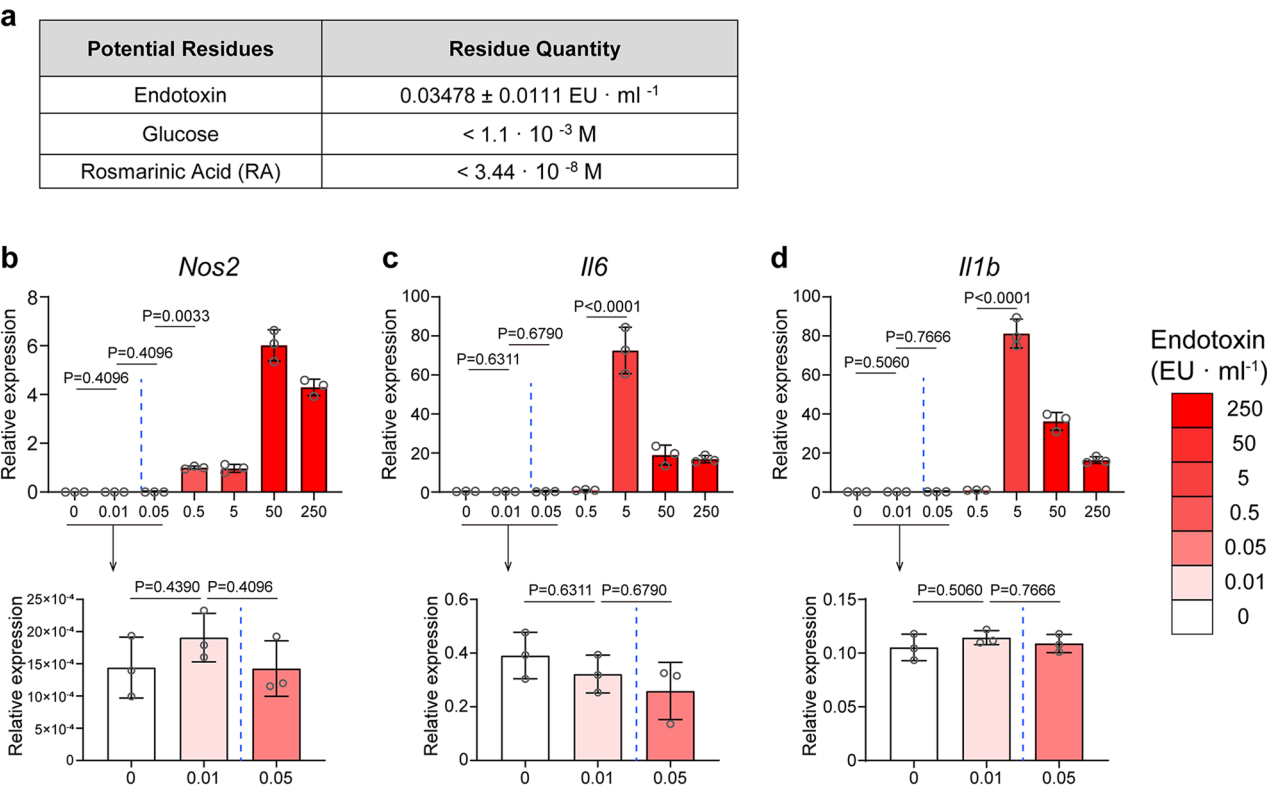
Extended Data Fig. 2 | MS/MS fragmentation spectra of standard crosslinking products. **a**, LOX crosslinking product, pyridinoline (PYD), is marked by green colour. **b**, TGM crosslinking product, γ -Glutamyl- ϵ -Lysine (γ -GLU- ϵ -LYS), is marked by red colour. **c-f**, AGE crosslinking products are marked by blue colour. Total crosslinking degree of AGE crosslinking is quantified by the molar

sum of CML (**c**), CEL (**d**), glucosepane (**e**), and pentosidine (**f**). The MH^+ labels indicate the protonated forms of precursor ions. Square labels above ions peaks are matched to their suggested fragment ion structures used to identify the crosslinking products.



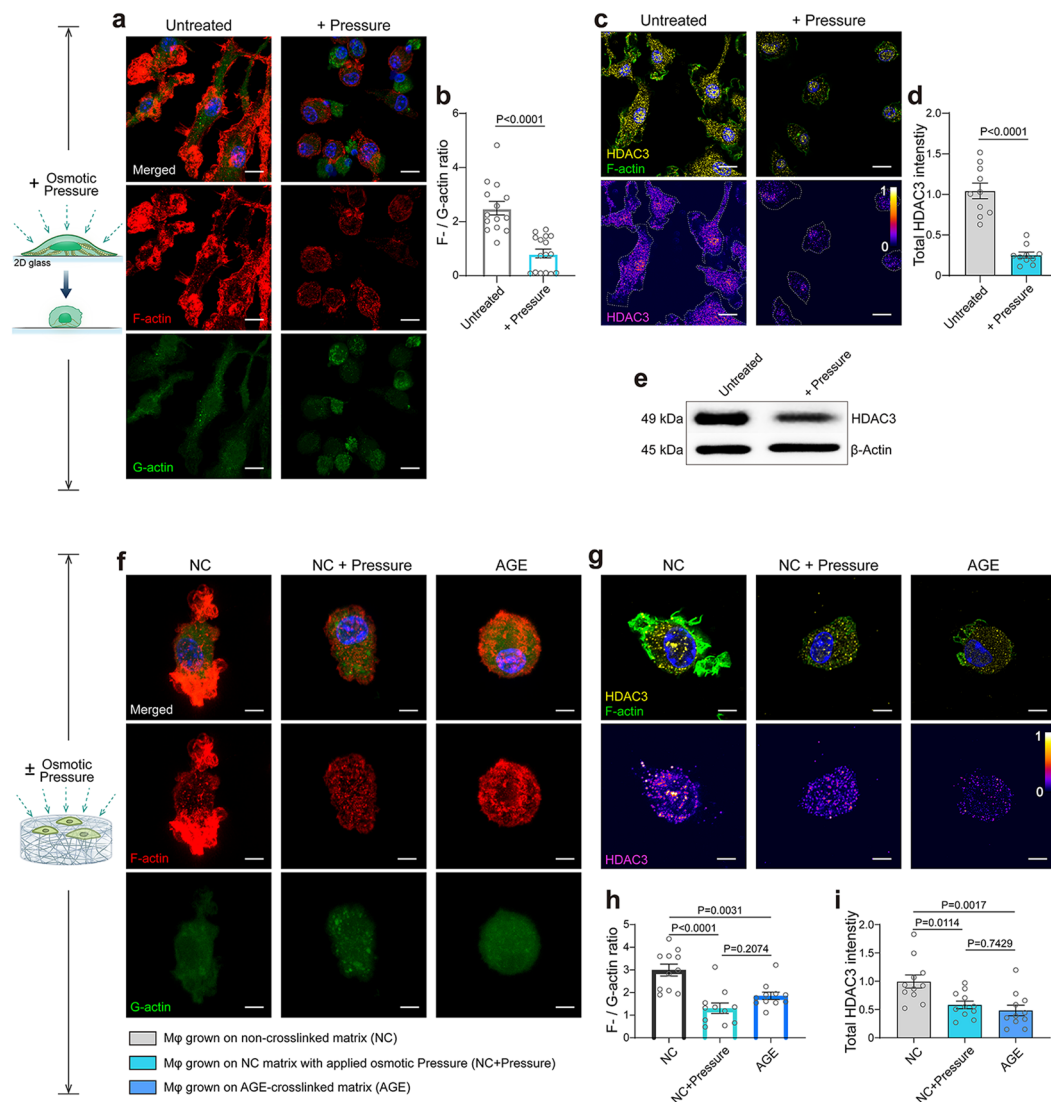
Extended Data Fig. 3 | Quantification of ECM crosslinking in livers from HFCDAA diet-induced liver fibrosis (HF fibrosis) model. **a**, Schematic of preparing liver decellularized ECM from mice with HFCDAA diet-induced liver fibrosis. **b**, Representative images of Sirius red staining in livers from healthy mice and HF-fibrosis mice. Scale bars, 200 μ m. **c**, Representative bright-field image of liver decellularized matrix harvested from HF-fibrosis mice. Scale bar, 1 cm. **d**, Representative SEM images of liver decellularized ECM from HF-fibrosis mice. Scale bars, 2 μ m. **e-j**, Quantification of the crosslinking degree in liver ECM from HF-fibrosis mice using AQMC method. Data are shown in mole quantity of

specific crosslinks normalized to mole quantity of tropocollagen molecules. **e**, LOX crosslinking degree quantified by pyridinoline ($n \geq 8$); **f**, TGM crosslinking degree quantified by γ -Glutamyl- ϵ -Lysine ($n \geq 8$); **g**, AGE crosslinking degree quantified by CML (**h**), CEL (**i**) and glucosepane (**j**) ($n \geq 8$); Pentosidine is not detectable in this HF-fibrosis model. Results are presented as means $\pm$ s.e.m. The statistical analysis was performed using two-tailed unpaired t-test. All n values represent pieces of liver decellularized matrix obtained from at least 4 biologically independent mice.



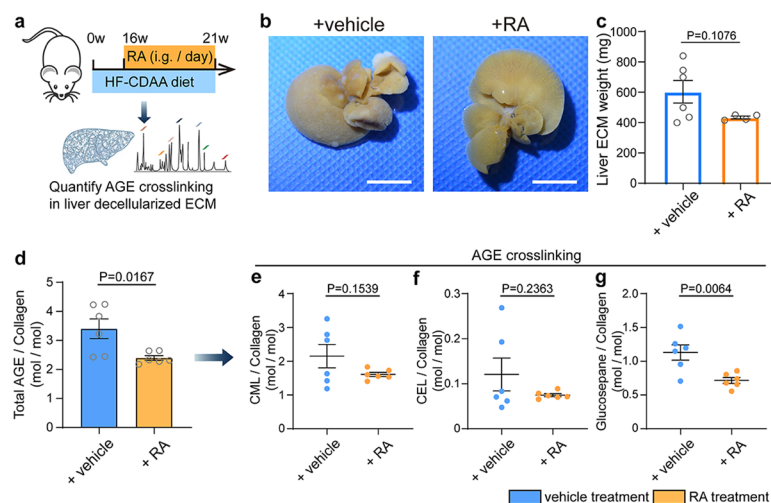
Extended Data Fig. 4 | Reagent residuals in the reconstructed AGE-crosslinked collagen matrix have negligible effects on macrophages' response. **a**, Characterization of reagent residuals in collagen matrix. **b**, Macrophages' response to endotoxin of different concentrations. Verified by relative mRNA expression of *Nos2* (**b**), *Il6* (**c**), and *Il1b* (**d**). The detailed results in upper panels within the endotoxin concentration range of 0–0.05 $\text{EU} \cdot \text{ml}^{-1}$ are shown in corresponding panels below ($n = 3$, biologically independent samples

per group). The blue dashed lines indicate the endotoxin residue levels in the reconstructed collagen matrix ($\sim 0.03 \text{ EU} \cdot \text{ml}^{-1}$). These results indicate that endotoxin residues in the *in vitro* collagen matrix keep at very low levels and cannot induce the unexpected response of macrophages. The statistical analysis was performed using a one-way ANOVA with Turkey test. Results are presented as mean $\pm$ s.d.



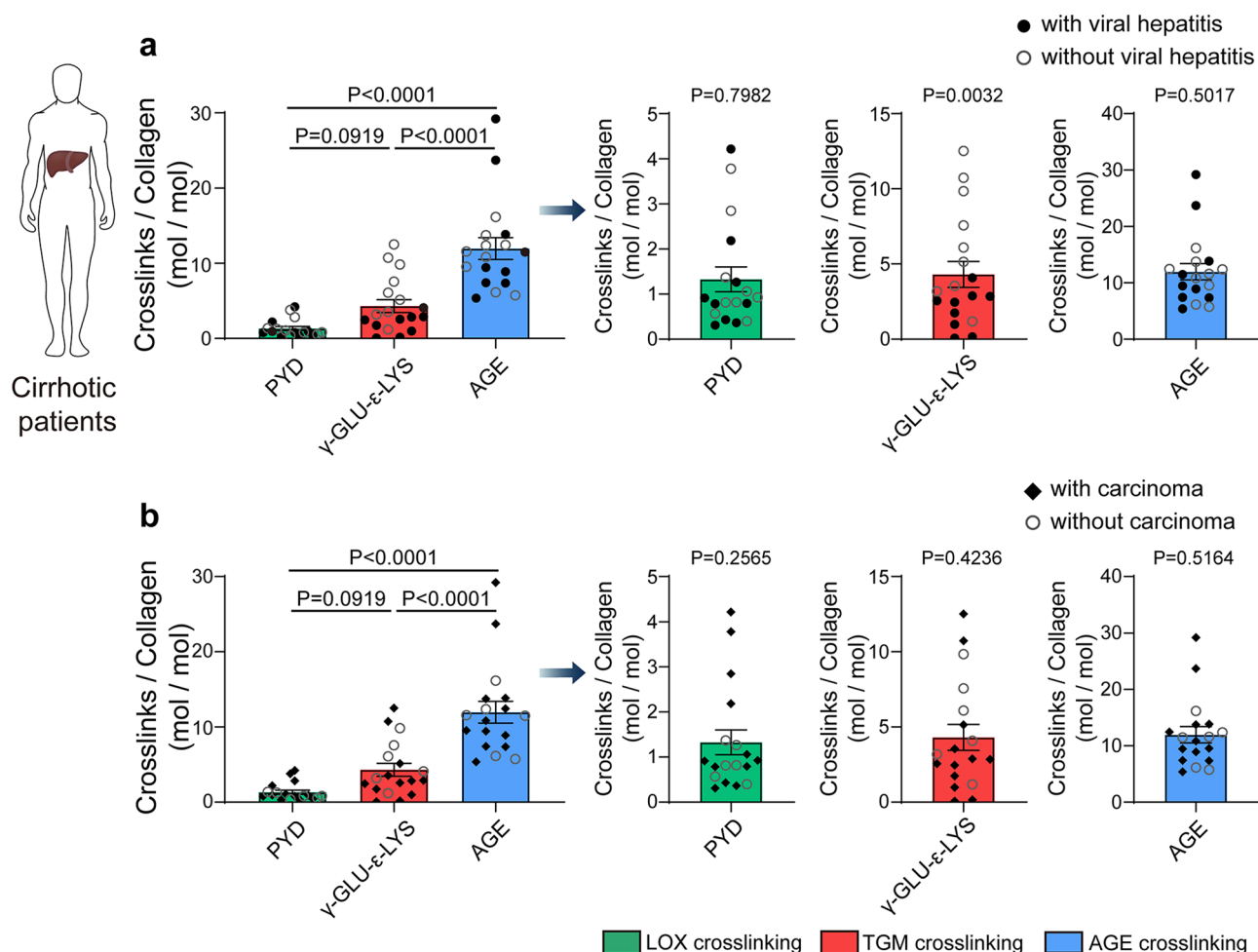
Extended Data Fig. 5 | Decreased expression of HDAC3 in macrophages is observed when inhibiting F-actin organization by applying increased osmotic pressure. **a-e**, Characterization of F-actin organization and HDAC3 expression in macrophages grown on 2D substrate. Cells were untreated or treated by PEG-induced osmotic pressure for 24 h (as detailed in *Methods* section). **a**, Representative images of F-actin (red) and G-actin (green) in macrophages with or without the treatment by the increased osmotic pressure. Scale bars, 10 μ m. **b**, Statistical analysis of F/G-actin ratio in macrophages as shown in (a) ($n \geq 14$, number of cells analysed per condition). **c**, Representative images of HDAC3 staining in macrophages. Top panel, HDAC3 (yellow), F-actin (green), cell nuclear (blue). Bottom panels, Colour-coded images of HDAC3. Colour bar indicates pixel intensity values. Scale bars, 10 μ m. **d**, Statistical analysis of total HDAC3 intensity per cell as shown in (c) ($n = 10$, number of cells analysed per condition). **e**, Western blot analysis of HDAC3 expression in macrophages. Data are representative of three independent experiments. **f-i**, Characterization of F-actin organization and HDAC3 expression in macrophages grown on

collagen matrix. NC (grey): macrophages grown on non-crosslinked collagen matrix; NC + Pressure (cyan): macrophages grown on non-crosslinked collagen matrix with treatment by PEG-induced osmotic pressure for 24 h; AGE (blue): macrophages grown on AGE-crosslinked collagen matrix. **f**, Representative images of F-actin (red) and G-actin (green) in macrophages grown on collagen matrix with or without the treatment by increased osmotic pressure. Scale bars, 4 μ m. **g**, Representative images of HDAC3 staining in macrophages. Top panel, HDAC3 (yellow), F-actin (green), cell nuclear (blue). Bottom panels, Colour-coded images of HDAC3. Colour bar indicates pixel intensity values. Scale bars, 4 μ m. **h**, Statistical analysis of F/G-actin ratio in macrophages as shown in (f) ($n = 11$, number of cells analysed per condition). **i**, Statistical analysis of total HDAC3 intensity per cell as shown in (g) ($n = 11$, number of cells analysed per condition). The statistical analysis was performed using two-tailed unpaired t-test in (b, d) and using a one-way ANOVA with Turkey test in (h, i). Results are presented as mean $\pm$ s.e.m.



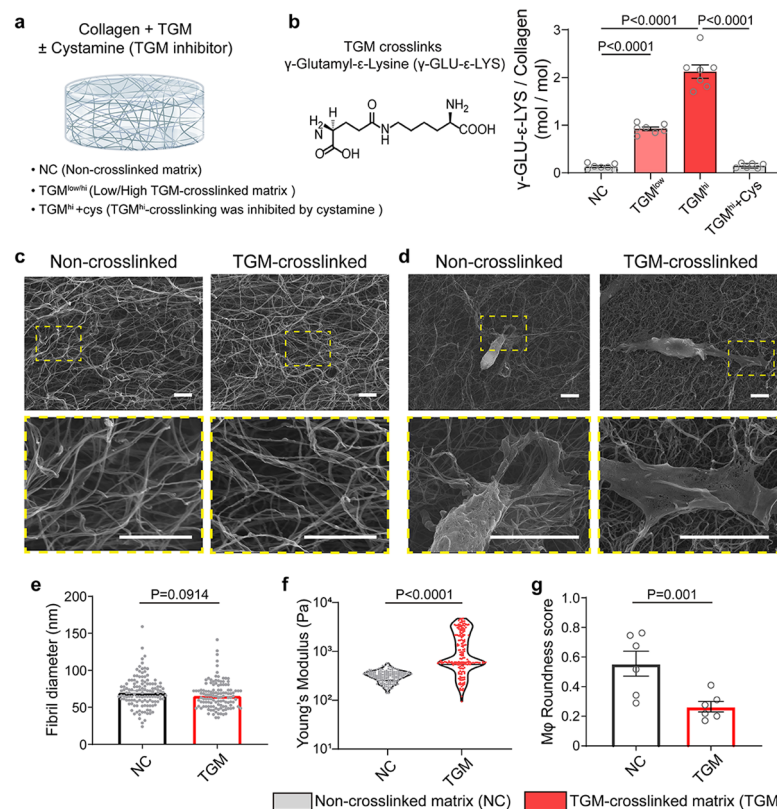
Extended Data Fig. 6 | Quantification of AGE crosslinking degree in liver ECM from mice with HFCDAA diet-induced liver fibrosis (HF fibrosis) after RA treatment. **a**, Schematic of quantifying AGE crosslinking in liver decellularized ECM from HF-fibrotic mice after RA treatment. **b**, Representative bright-field images of liver decellularized ECM. Scale bars, 1 cm. **c**, Statistical analysis of the dry weight of entire liver decellularized ECM ($n \geq 4$, biologically

independent mice per group). **d-g**, Quantification of AGE crosslinking degree of liver decellularized ECM using AQMC method. **d**, Total AGE crosslinking degree; **e**, CML; **f**, CEL; **g**, Glucosepane ($n = 6$, pieces of liver decellularized ECM derived from at least 3 biologically independent mice). Results are presented as mean $\pm$ s.e.m. The statistical analysis was performed using two-tailed unpaired t-test.



Extended Data Fig. 7 | Comparative analysis of ECM crosslinking degree from cirrhotic patients' liver samples with different disease history. a, Results from cirrhotic patients with ($n = 9$, pieces of liver ECM obtained from 5 independent patients) or without ($n = 9$, pieces of liver ECM obtained from 4 independent patients) viral hepatitis. **b,** Results from cirrhotic patients with ($n = 12$, pieces of

liver ECM obtained from 6 independent patients) or without ($n = 6$, pieces of liver ECM obtained from 3 independent patients) carcinoma. The statistical analysis was performed using two-tailed unpaired t-test between the groups with or without viral hepatitis (**a**), and with or without carcinoma (**b**). Exact P values are marked on the plots. Results are presented as mean $\pm$ s.e.m.



Extended Data Fig. 8 | TGM crosslinking affects structural and mechanical properties of collagen matrix in a way different from AGE crosslinking. a, Schematic of reconstructing TGM-crosslinked collagen matrix *in vitro*. NC, Non-crosslinked collagen matrix; TGM^{low}, TGM-crosslinked collagen matrix with a low crosslinking degree; TGM^{hi}, TGM-crosslinked collagen matrix with a high crosslinking degree; TGM^{hi} + cys, collagen matrix with additional cystamine to inhibit TGM^{hi} treatment. **b**, Quantification of TGM crosslinking degree of reconstructed collagen matrix using AQMC method. Data are shown in mole quantity of γ-Glutamyl-ε-Lysine normalized to mole quantity of tropocollagen molecules ($n \geq 6$, independent collagen matrix samples). TGM^{hi} collagen matrix is selected for the following assays in **c-g** (shown as TGM-crosslinked). **c**, Representative SEM images of non-crosslinked and TGM-crosslinked collagen

matrix. Scale bars, 2 μ m. **d**, Representative SEM images of macrophages grown on non-crosslinked and TGM-crosslinked collagen matrix. The bottom panels show protrusions of macrophages binding adjacent collagen fibrils. Scale bars, 5 μ m. **e**, Statistical analysis of fibril diameter ($n \geq 123$, number of fibrils randomly selected from at least 5 fields of SEM images). **f**, Statistical analysis of the Young's modulus of non-crosslinked and TGM-crosslinked collagen matrix measured by AFM ($n \geq 171$, points of measurement randomly selected from at least 10 fields of samples). **g**, Statistical analysis of the roundness of macrophages grown on non-crosslinked and TGM-crosslinked collagen matrix ($n = 6$, number of cells analysed per condition). The statistical analysis was performed using a one-way ANOVA with Turkey test in (**b**) and using two-tailed unpaired t-test in (**e-g**). Results are presented as mean $\pm$ s.e.m.

Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☒ ☐ A description of all covariates tested
- ☐ ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection Olympus FV3000 FV31S-SW version 2.3.2.169 (to obtain confocal images)
AB SCIEX Analyst version 1.7.1 (to obtain HPLC-MS/MS data)
SerialEM version 3.7.13 (to obtain Cryo-EM data)
DigitalMicrograph version 3.32.2403.0 (to obtain Cryo-EM data)
Pannoramic scanner version 1.23 (to obtain histological stained images)
Details are described in Methods, the relevant figure legends and the Supplementary information.

Data analysis Graphpad Prism version 8 (for statistical analysis)
Imaris version 9.6.0 (for the analysis of confocal images)
Adobe Illustrator version 2020 (for figure preparation)
Flowjo version 10 (for analysing flow-cytometry data)
Amira version 20.2 (for processing Cryo-EM data)
IMOD software package version 4.9.12 (for analysing Cryo-EM data)
R package pheatmap version 1.0.12 (for analysing RNA-seq data)
Details are described in Methods, the relevant figure legends and the Supplementary information.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The main data supporting the results in this study are available within the paper and its Supplementary Information. Source data are provided with this paper. The raw RNA-seq data are available at the SRA database via the accession numbers PRJNA852213 and PRJNA856261.

Human research participants

Policy information about [studies involving human research participants and Sex and Gender in Research](#).

Reporting on sex and gender	The sex and the gender of the participants is provided in Supplementary Table 1.
Population characteristics	Provided in Supplementary Table 1.
Recruitment	Eligible patients were consented to participate in the study and to the tissue-sample-collection protocol. Informed consents were obtained from all participants. No apparent recruitment biases were present.
Ethics oversight	Institutional Review board of Tsinghua University.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample sizes were determined on the basis of preliminary experiments and from previously published results, so as to provide sufficient statistical power to discern statistically significant differences.
Data exclusions	No exclusion criteria were established in this study. One RNA sample was excluded from sequencing owing to the poor RNA quality (excluded sample: AGE_3, AGE group in type-I immune-response evaluation).
Replication	All experiments were replicated and were conducted with both biological and technical replicates, with similar results.
Randomization	For the in vitro experiments, prepared collagen matrices were randomly selected to seed cells for the following assays. For the in vivo experiments, mice were randomly distributed across groups before CCI4 and HFDCAA diet induction. The mice with established liver fibrosis were redistributed into different groups before drug treatment.
Blinding	No blinding was used for the in vitro experiments because the data analyses were based on objectively measurable data, and because standardized procedures for data collection and analysis were used to prevent bias. For the in vivo experiments, data acquisition was performed blindly, and researchers were unblinded during data analysis because the data analyses were based on objectively measurable data, and because standardized procedures for data collection and analysis were used to prevent bias.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

anti-LOX (Abcam, Cat# ab31238, dilution 1:200, Polyclonal, immunofluorescence (IF), mouse/human tissue)
 anti-Transglutaminase 2 (Abcam, Cat# ab2386, dilution 1:200, Monoclonal, Clone CUB 7402, IF, mouse/human tissue)
 anti-AGE (Abcam, Cat# ab23722, dilution 1:200, Polyclonal, IF, mouse/human tissue)
 anti-COL-1 (Abcam, Cat# ab6308, dilution 1:200, Monoclonal, Clone COL-1, IF, mouse/human tissue)
 anti-αSMA (eBioscience, Cat# 14-9760-82, dilution 1:400, Monoclonal, Clone 1A4, IF, mouse/human tissue)
 anti-Paxillin (Abcam, Cat# ab32115, dilution 1:200, Monoclonal, Clone E228, IF, macrophage)
 anti-HDAC3 (Abcam, Cat# ab32369, dilution 1:200, Monoclonal, Clone Y415, IF, macrophage)
 anti-CD45 BV605 (Biolegend, Cat# 103155, dilution 1:100, Monoclonal, Clone 30-F11, Flow cytometry, macrophages analysis)
 anti-ly6G Alexa Fluor 488 (Biolegend, Cat# 127626, dilution 1:100, Monoclonal, Clone 1A8, Flow cytometry, macrophages analysis)
 anti-CD11b BV421 (Biolegend, Cat# 101251, dilution 1:100, Monoclonal, Clone M1/70, Flow cytometry, macrophages analysis)
 anti-F4/80 PE-Cy7 (Biolegend, Cat# 123114, dilution 1:100, Monoclonal, Clone BM8, Flow cytometry, macrophages analysis)
 anti-Arginase-1 APC (eBioscience, Cat# 17-3697-82, dilution 1:100, Monoclonal, Clone A1exF5, Flow cytometry, macrophages analysis)
 anti-RELMα PE (eBioscience, Cat# 12-5441-80, dilution 1:100, Monoclonal, Clone DS8RELM, Flow cytometry, macrophages analysis)
 anti-iNOS APC (eBioscience, Cat# 17-5920-80, dilution 1:100, Monoclonal, Clone CXNFT, Flow cytometry, macrophages analysis)
 anti-IL-6 PE (Biolegend, Cat# 504504, dilution 1:100, Monoclonal, Clone MP5-20F3, Flow cytometry, macrophages analysis)
 anti-CX3CR1 PE (Biolegend, Cat# 149005, dilution 1:100, Monoclonal, Clone A011F11, Flow cytometry, macrophages analysis)
 anti-Tgfβ1-LAP APC (Biolegend, Cat# 141405, dilution 1:100, Monoclonal, Clone TW7-16B4, Flow cytometry, macrophages analysis)
 anti-HDAC3 (Cell Signaling Technology, Cat# 85057S, dilution 1:1000, Clone D2O1K, Western blot)
 anti-β-Actin (Cell Signaling Technology, Cat# 4970S, dilution 1:2500, Clone 13E5, Western blot)

Validation

All commercially available antibodies have been thoroughly tested and validated by the manufacturers. All the antibodies have been validated to be suitable for the corresponding application in this study. Validation statements are available on the manufacturers' websites.

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)	The human hepatic stellate cell line (LX-2) was bought from Xiangya Hospital of Centre-South University.
Authentication	LX-2 was characterized by SMA, DDR2 and Vimentin staining.
Mycoplasma contamination	The cell lines tested negative for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used.

Animals and other research organisms

Policy information about [studies involving animals; ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	C57BL/6 8-weeks-old male mice were used.
Wild animals	The study did not involve wild animals.
Reporting on sex	Only male animals were used. Sex stratification in animal experiments was not considered in this study. In our review of the literature regarding liver-fibrosis models, there was lack of solid evidence indicating bias or of differences when female or male mice were used.
Field-collected samples	The study did not involve samples collected from the field.
Ethics oversight	All the animal protocols used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Tsinghua University

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

- ☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☒ All plots are contour plots with outliers or pseudocolor plots.
- ☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

For BMDM, cells were harvested from collagen matrix by degrading the matrix using collagenase. For primary hepatic macrophages, a single-cell population was obtained by sequential enzymatic digestion of liver tissue. Sample-preparation details are described in Methods.

Instrument

BD LSRFortessa SORP

Software

BD FACSDiva

Cell population abundance

Cell counts of 10,000 or 1,000,000 events were collected for a relevant cell population after initial gating for BMDM and primary hepatic cells, respectively.

Gating strategy

Figures exemplifying step-by-step gating strategies are provided in the Supplementary Information. Cells were firstly gated by FSC-A/FSC-H and SSC-A/SSC-W, followed by CD11b+/F480+ gating for macrophages. The negative population was determined via an unstained cell control.

- ☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.